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INVESTIGATIONS ON THE ROLE OF THE CENTRAL NERVOUS SYSTEM IN SHOCK PRODUCED BY ENDOTOXIN FROM GRAM-NEGATIVE MICROORGANISMS

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THE reactions produced by endotoxin from various species of gram-negative organisms are strikingly similar.[1, 2] A variety of reproducible functional and anatomic disturbances follow the intravenous administration of such bacterial products to animals. Our observations in the dog following the administration of endotoxin have confirmed those of many other investigators with respect to the development of profound shock, fever, neutrophilic leukopenia and then leukocytosis, thrombocytopenia, hyperglycemia followed by hypoglycemia, and extensive pathologic changes in the gastrointestinal tract.[3-7] A similar clinical picture, but not including the extensive pathologic alterations, has been reported in human cases following the accidental administration of blood contaminated with a heavy inoculum of gram-negative microorganisms.[8-10]

When the sequence of events following administration of endotoxin had been well established for the anesthetized animal, our attention was directed to the role of the central nervous system in the causation of shock. In view of earlier reports, it was of particular interest to determine whether endotoxin acted in some way on higher nervous centers which in turn set off a mechanism terminating in irreversible shock. As early as 1872, Dubczanski and Naunyn[11] suggested, on empirical grounds, that pyrogenic substances produce fever and "vasomotor collapse" through a central action on the vasomotor center. Charrin

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and Gley,[12] in 1890, produced shock in the rabbit by the intravenous administration of crude pyocyaneus endotoxin and found that shocked animals displayed a deficient response to vasopressor and vasodepressor nerve stimulation. They postulated that this was a consequence of central (medullary) vasomotor paralysis. Romberg and associates[13] reviewed the literature up to 1899 and reported results of experimental attempts to show that it was not cardiac weakness per se that led to shock in acute infectious disease. Excluding a primary cardiotoxic action, these authors held that "central vasomotor collapse" was the responsible factor but presented no further evidence in support of this postulation. As an outgrowth of these early theories, the concepts of shock and vasomotor collapse have become synonymous and the impression that these result from a primary action of toxic products on the vasomotor center has persisted.

Penner and Klein[14] gave further support to this concept in 1952 when they reported that certain systemic effects of toxin from Shigella were mediated through a direct effect on the brain. These authors attempted to isolate the head and body circulations of the dog by a cross-circulation technique. The head of Dog 1 was perfused through a carotid artery and an external jugular vein of Dog 2, and vice versa for Dog 2. The vertebral arteries and veins and the second carotids and jugulars of both dogs were ligated. In six pairs of dogs under pentobarbital anesthesia, they injected Shiga toxin into the femoral vein of one dog of each pair and reported that only the dog whose head was supplied with blood from the body of the injected dog developed leukopenia, hyperglycemia, hemoconcentration, and gastrointestinal lesions similar to those induced in intact dogs. They accordingly concluded that Shiga toxin acts primarily on the brain and further suggested that the toxin acts directly on the hypothalamus. Postulating that a diffuse sympathomimetic discharge is precipitated, the authors further supposed that this in turn causes the typical physiologic and pathologic changes. This work has been cited by others as evidence that endotoxins from gram-negative bacteria as a group exert their effects through a central nervous system action.[15, 16] The present studies were designed to reinvestigate the possible role of the central nervous system in the production of shock induced by these endotoxins.

METHODS

I. *Endotoxin.*—Purified *Escherichia coli* and *Brucella melitensis* endotoxins of the Boivin type were employed in most experiments. The bacteria were seeded on "Albimi" agar, and endotoxin was extracted from the bacterial bodies harvested after 18 to 24 hours' growth by a method previously described.[17] Preliminary trials showed that the intravenous injection of 5 mg. per kilogram of *E. coli* endotoxin or 10 mg. per kilogram of *Br. melitensis* endotoxin were just adequate to produce shock regularly in the dog. Preliminary toxicity studies in the ABC strain of white mice* weighing 15 to 20 milligrams showed that 1 mg. of the Brucella endotoxin or 0.4 mg. of the *E. coli* endotoxin was lethal for 80 to 100 per cent of mice following intraperitoneal injection. Because of the large quantities which were necessary to produce shock in the dog, a suspension of heat-killed *E. coli* bacteria was used for some experiments. Response to this "crude endotoxin" was identical to the purified preparation in terms of arterial blood pressure, portal vein pressure, and pathologic change. This endotoxin was produced from cells grown and harvested in the same manner as purified endotoxin. The cells were washed, resuspended in an equal volume of saline, and trans-

*Obtained from Dr. John Bittner at the University of Minnesota.

ferred to 20 ml. vials containing glass beads for effective agitation. These vials were then placed in a water bath of 56° C. for 1 hour with frequent shaking. The "crude" endotoxin so produced was lethal for 80 per cent of mice after intraperitoneal injection of 0.07 ml. Shock was invariably produced in mongrel dogs by intravenous injection of 0.2 to 0.3 ml. of crude endotoxin per kilogram of body weight.

II. *Procedure.*—In initial experiments, dogs were trained to lie on their sides on a table. Blood pressures were measured indirectly by use of a small cuff and an infant stethoscope with a miniature diaphragm according to a method described by Cannon.[18] Purified endotoxin from *Br. melitensis* or *E. coli* was injected intravenously. Subsequent experiments were done with sodium pentobarbital administered intravenously in a dosage of 30 mg. per kilogram with supplementary doses as needed. Puppies were avoided because shock could not be consistently produced in the immature animal. In anesthetized animals, blood pressures were measured by use of a strain gauge and direct-writing Sanborn Poly-Viso. Arterial pressures were measured in the aorta through a catheter advanced from the femoral or carotid artery. Portal vein pressure was measured by a catheter advanced into it through a splenic branch vein. Endotoxin was injected into the femoral or the jugular vein. In the experiments with dogs, the amount injected in each case was 10 mg. per kilogram purified *Br. melitensis* endotoxin, 5 mg. per kilogram purified *E. coli* endotoxin, or 0.3 ml. "crude" *E. coli* endotoxin. A series of preliminary observations demonstrated that the effects were approximately the same following the administration of these quantities in mice and in dogs. Most of the animals were artificially respired with air through a tracheal cannula.

Cerebral cross circulation was performed successfully in three paired dogs according to the method of Penner and Klein.[14] In one of the experiments, the vertebral arteries were tied off at the point of inflow into the vertebral canal only one hour before the injection of endotoxin. The method differed in one important respect from that of Penner and Klein in that the head was perfused through both carotid arteries and jugular veins. Purified *E. coli* endotoxin, the dosage of which was calculated on the basis of the body weight of the larger animal, was injected into the femoral vein of the smaller animal.

In four dogs, the spinal cord was ligated and transected at the level of the sixth cervical vertebra, 18 hours before the injection of endotoxin. In one animal, chordotomy was immediately followed by injection of endotoxin. Both vagus nerves were transected high in the neck shortly before the administration of endotoxin in two animals. Chordotomy and the acute experiment were both done under pentobarbital anesthesia, except that one animal was not anesthetized at the time of injection of endotoxin.

Three dogs were surgically decapitated at the level of the sixth cervical vertebra. A fall in blood pressure, in part due to a loss of blood, was effectively counteracted by the administration of blood or dextran solution. Endotoxin was injected into the femoral vein after blood pressure had been stable over a satisfactory control period.

The effect of crude and refined *E. coli* endotoxin on the arterial and portal vein pressure of the cat was studied in anesthetized animals weighing between 2.3 and 3.6 kilograms. Thirty mg. per kilogram sodium pentobarbital were given intraperitoneally and recording methods were identical to those described for the dog. After preliminary tracheotomy and mechanical respiration, a small incision was made in the posterior midline over the atlanto-occipital junction, the neck was acutely flexed, and a pliable metal rod was guided into the spinal canal. Correct insertion was assured by observation of tonic contraction followed by flaccidity as the rod was advanced caudally. Two pithed, two pithed-vagotomized, and five unoperated control animals were studied. Purified *E. coli* endotoxin in a dose of 5 mg. per kilogram in two cats and 10 mg. per kilogram in two additional cats and crude *E. Coli* endotoxin in the remaining animals were employed.

RESULTS

I. *Intact Dogs.*—As soon as the injection of endotoxin was completed, the unanesthetized animal had a forceful, well-formed bowel movement. Within two to three minutes, hyperpnea, agitation, vomiting, and diarrhea occurred. The animals began to shiver markedly, the systemic blood pressure fell pro-

TABLE I. REPRESENTATIVE ARTERIAL BLOOD PRESSURE, PORTAL VEIN PRESSURE, AND PATHOLOGIC CHANGES PRODUCED BY ENDOTOXIN

NO.	WT.	SEX	ENDOTOXIN	MEAN ARTERIAL PRESSURE IN MM. HG			PORTAL VEIN PRESSURE RISE (MM. HG)	SURVIVAL TIME IN HOURS	PATHOLOGY
				BEFORE	IMMED.	1 HR. PRE-MORTAL			
1	13.6	M	Brucella (purif.)	130	18	40 35	55	6¾*	Severe enterie hemorrhage. Slight gall-bladder edema
2	8.4	F	Brucella (purif.)	125	60	60 90	12	5¾*	Severe enterie hemorrhage. Severe gall-bladder edema
3	12.2	F	E. coli (purif.)	130	95	45 85	6	5¾*	Severe enterie hemorrhage. Severe gall-bladder edema. Bilateral adrenal hemorrhage
4	12.0	M	E. coli (purif.)	125	35	85 85	15	1½*	Submucosal congestion of intestine. Moderate gall-bladder edema
5	7.0	M	E. coli (crude)	160	30	130 32	7	4½	Submucosal congestion of intestine. Severe hemorrhagic gall-bladder edema
6	20.0	M	E. coli (crude)	106	30	60 38	8	6¾*	Severe enterie hemorrhage. Pseudomembrane in stool†
7	14.0	M	E. coli (crude)	130	33	25 15	10	1	Severe enterie hemorrhage. Severe gall-bladder edema

*Sacrificed.

†A second equivalent dose of endotoxin, given after 3 hours, caused a fall in mean arterial pressure from 62 to 40 mm. Hg. and a rise of portal pressure of 5 mm. Hg.

gressively and the animals became apathetic. Frequently, they would walk in an ataxic fashion for short distances, dragging their hind legs as if paralyzed. Over the first hour, gagging and tenesmus gave way to profuse bloody vomitus and bloody diarrhea. In some animals, strands of material resembling the

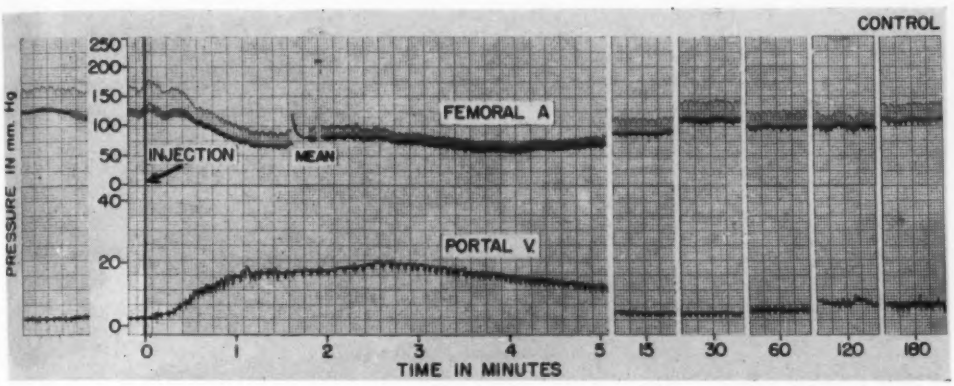

Fig. 1.—Arterial and portal pressure curves following the administration of purified *E. coli* endotoxin to the intact dog.

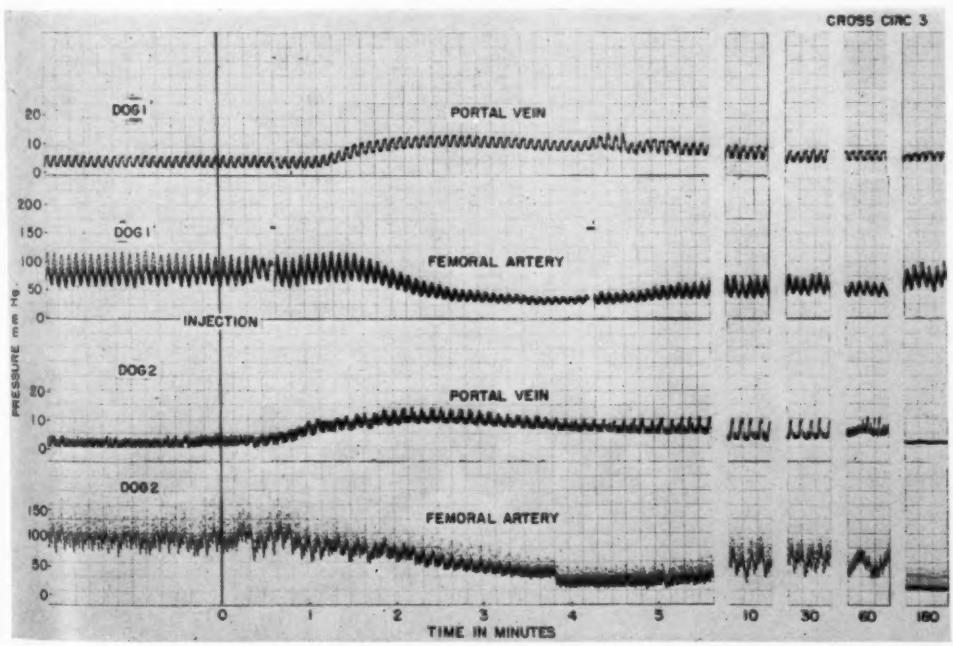

Fig. 2.—Arterial and portal pressure changes following injection of purified *E. coli* endotoxin into Dog 2 in a pair of dogs united by cerebral cross circulation.

mucosa were extruded from the rectum in the form of a pseudomembrane. Coma ensued in three and death within four to eighteen hours. On autopsy, the most prominent features were severe congestion of the liver, submucosal bleeding, and surface epithelial slough of large portions of the small intestine. The duodenum was most severely affected, and marked edema of the gallbladder wall was a constant feature.

TABLE II. PERIPHERAL HEMATOLOGIC VALUES AND GLUCOSE DETERMINATIONS IN CROSS-CIRCULATED DOGS

TIME	W.B.C.		PMN (%)		NUCLEATED R.B.C. PER 100 W.B.C.		HEMOGLOBIN (GM. %)		HEMATOCRIT (VOL. %)		BLOOD SUGAR (GM. %)	
	DOG 1	DOG 2	DOG 1	DOG 2	DOG 1	DOG 2	DOG 1	DOG 2	DOG 1	DOG 2	DOG 1	DOG 2
After anesthesia	22,350	14,850	78	60	0	0	12.9	13.2	41	41.5	88	82
After cross circulation	6,050	9,350	72	71	0	0	15.3	15.4	50.5	50	156	97
0 Hour	*Injection of E. coli Endotoxin Into Dog 2*											
1 Hour	4,500	4,500	63	64	17	38	14.7	15.1	48	48	91	72
2 Hours	6,950	7,350	80	82	19	25	14.4	14.5	47	46.5	68	68
3 Hours	18,200	17,550	81	88	12	11	14.3	14.8	47	47	56	90

TABLE III. THE EFFECT OF ENDOTOXIN WHEN ADMINISTERED TO CHORDOTOMIZED DOGS

NO.	WT.	SEX	ENDOTOXIN	MEAN ARTERIAL PRESSURE IN MM. HG				PORTAL VEIN PRESSURE RISE (MM. HG)	SURVIVAL TIME IN HOURS	REMARKS
				BEFORE	IMMED.	1 HR.	PRE-MORTAL			
1	5.6	F	Brucella (purif.)	78	95	50	45	5	1¾	Puppy; not respirated; had asphyxial death
2	9.2	M	Brucella (purif.)	135	30	40	20	15	3¾	--------
3	15.4	M	E. coli (purif.)	70	25	37	25	4	1½	Vagotomy 20 minutes before injection of endotoxin
4	14.5	M	E. coli (purif.)	145	38	65	35	--	1½	Spinal transection and vagotomy done acutely. Received 200 ml. isotonic saline during procedure
5	20.0	M	E. coli (purif.)	75	60	45	45	7	1	No anesthesia; not respirated; had asphyxial death

After anesthesia, the endotoxin no longer caused any observable gastrointestinal signs and hyperpnea was minimal. A precipitate fall of the arterial pressure to shock levels occurred within one minute after injection, and it was found that the portal vein pressure increased up to four times within the first five minutes (Fig. 1). The portal pressure decreased to its former value and there was partial recovery of the arterial pressure during the first 15 minutes. Over the subsequent 1 to 5 hours the arterial pressure again fell to lower values from which it did not ordinarily recover. Typical gross pathologic changes indistinguishable from those of unanesthetized animals were found upon autopsy (Table I). There was no apparent difference in the hemodynamic and pathologic effects produced by the three types of endotoxin.

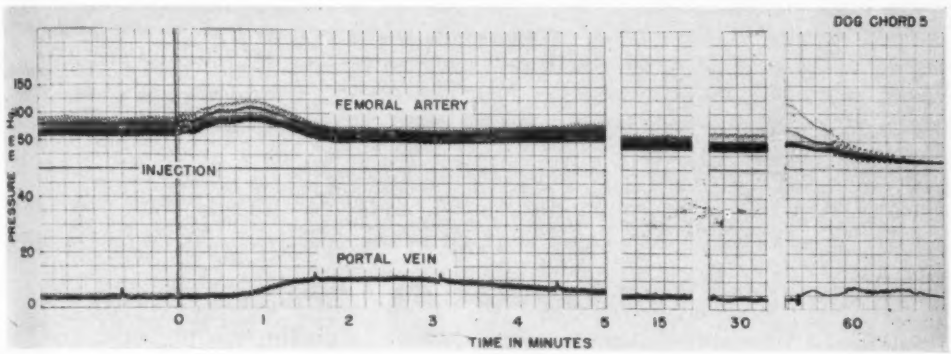

Fig. 3.—Femoral artery and portal vein pressure response in an unanesthetized, chordotomized dog. The animal was not respirated, and asphyxial death occurred at the end of one hour.

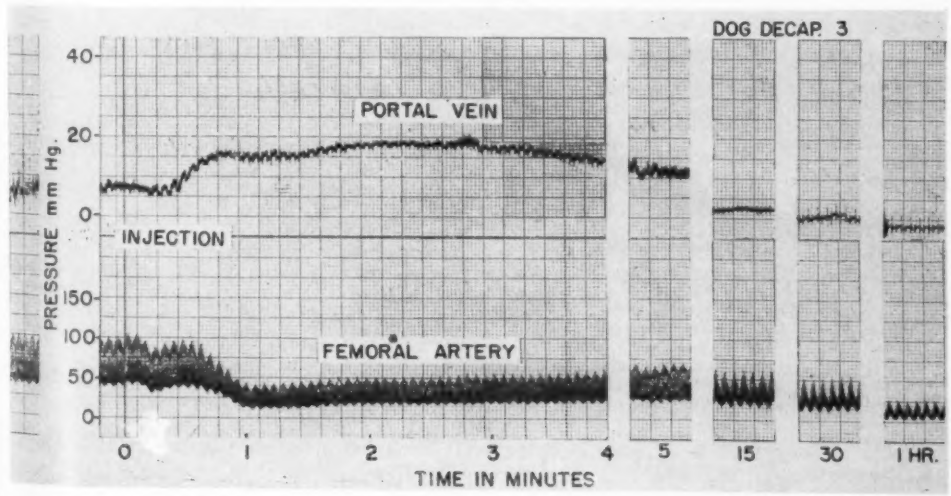

Fig. 4.—Arterial and portal vein pressure changes in the decapitated dog preparation.

II. *Cerebral Cross Circulation.*—In each experiment both dogs displayed the characteristic arterial and portal vein pressure changes (Fig. 2). There was no evidence that either dog was protected and, contrary to expectation, pressure changes occurred slightly earlier in the dog into whose body the endotoxin was injected. Pathologic changes in the gastrointestinal tract and gall

TABLE IV. EFFECTS OF ENDOTOXIN IN DECAPITATED DOGS

NO.	SEX	ENDOTOXIN	MEAN ARTERIAL PRESSURE IN MM. HG		PORTAL VEIN PRESSURE RISE IN MM. HG	SURVIVAL (MINUTES)	COMMENTS
			CONTROL	IMMEDIATE FALL			
1	M	E. coli (crude)	75	25	6	25	Abrupt blood pressure fall without recovery
2	M	E. coli (crude)	185	50	8	13	Abrupt blood pressure fall. Given second dose of endotoxin at 13 minutes causing another sharp fall and death in one minute
3	F	E. coli (crude)	85	25	12	75	B. P. returned to 45 mm. Hg at 6 minutes and then progressively declined until death. Characteristic intestinal and gallbladder changes on autopsy

bladder of equivalent severity were seen on autopsy of each dog. Hemoglobin, hematocrit, white blood count, differential, and blood glucose were essentially similar in each dog of the pair in contrast to the difference reported by Penner and Klein (Table II).

III. *Chordotomy.*—Chordotomy with or without vagotomy did not prevent the fall in arterial blood pressure, the elevation in portal vein pressure, and the characteristic morphologic changes (Table III). An initial rise in arterial pressure followed by a steep fall was observed in some animals (Fig. 3). The survival time of chordotomized dogs was significantly decreased.

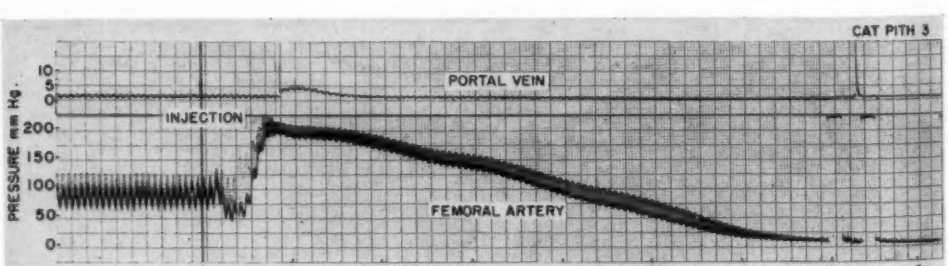

Fig. 5.—Arterial and portal pressure changes following the injection of crude *E. coli* endotoxin into a vagotomized cat whose spinal cord had been pithed. The animal had a tracheotomy and was respirated on air.

IV. *Decapitation.*—The decapitated preparation also displayed the immediate blood pressure fall and portal vein elevation characteristic of endotoxin shock (Table IV and Fig. 4). Since death occurred in less than one hour, typical morphologic changes were seen in only one preparation which survived for 75 minutes.

V. *Mechanical Destruction of the Spinal Cord.*—The response of intact cats to endotoxin was variable. Blood pressure usually was elevated as an immediate response and this was promptly followed by a more gradual decline, over several minutes, to shock levels. Portal pressure increased only 1 to 3 mm.

and pathologic changes similar to those of the dog were observed in about half of the animals studied. Spinal cord destruction with or without vagotomy did not prevent shock due to endotoxin. In the pithed animals, there was no recovery from the sharp blood pressure drop, so that death occurred soon after the injection (Fig. 5).

DISCUSSION

The cerebral cross-circulation experiments of Penner and Klein were performed with *Shigella dysenteriae* toxin. While the symptoms that are produced by most endotoxins from gram-negative organisms are more or less the same regardless of the species from which the endotoxin is derived, Shiga toxin is not typical in this regard.[1] Unlike *E. coli* and *Br. melitensis, S. dysenteriae* forms a neurotoxin which has properties of an exotoxin.[19] This neurotoxin may be responsible for primary central nervous system effects. On the other hand, the present cross-circulation experiments using typical endotoxins from gram-negative bacteria do not support the widely quoted generalization that endotoxin primarily attacks the brain. Braude and associates[16] were unable to detect significant localization of toxin in the brain of rabbits receiving lethal doses of radioactive *E. coli* endotoxin, and their recent experiments are of interest in view of the present findings.

The close correspondence of changes observed in each member of the cross-circulated pair is not surprising in view of the extensive interchange of blood that can occur through anastomotic channels in skin, muscle, and especially a rich vascular network surrounding the spinal cord. In numerous other experiments we found that a large amount of blood can reach the head through channels within the spinal canal. In one experiment the head of one large dog was entirely separated from the body, except that the vertebral column was left intact. The head was perfused through the carotid arteries and jugular veins by a mechanical pump-oxygenator at normal pressure. When red blood cells labeled with radioactive chromium (Cr^{51}) were injected into the cerebral circulation, the specific activity of blood in the body circulation (femoral vein) was more than 13 per cent of the activity in the cerebral circulation at the end of five minutes. After the animal had been sacrificed, the cephalic circulation was perfused with 500 ml. of a barium suspension. Subsequent x-ray study showed that intraspinal connections were so extensive that there was retrograde filling of upper intercostal vessels with contrast medium. In the intact Penner-Klein preparation, skin and muscle allowed for an even greater interchange of blood. The amount of blood that crosses through anastomotic channels must indeed be large because in several of our experiments, animals continued to respire spontaneously for as long as 35 minutes, although the heart of the opposite dog which presumably supplied blood to the brain of the survivor had stopped. The medullary respiratory center was evidently supported by anastomotic channels since both carotids and vertebrals had been transected. Through the same anastomotic channels, significant amounts of endotoxin presumably entered the opposite circulation.

It does not appear likely that the differences in results between those of Penner and Klein and the present cross-circulation experiments are accounted

for by differences in the amount of toxin injected. In order to achieve a consistent response, an excess of toxin was used in both studies. Further clarification of quantitative dosage factors is difficult in experiments with mongrel dogs because of the great variability in the intensity of response from one animal to another.

In the course of further inquiry into the nature of shock due to endotoxin, we have found that the injection of endotoxin causes extensive venous pooling in the dog.[20] This leads to a deficiency in venous return, a decreased cardiac output, and therefore, a fall in arterial blood pressure. The gastrointestinal venous pool, particularly that of the liver, acts as a storage reservoir so that the portal vein pressure is elevated and extensive pathologic alterations are produced in the viscera. If the liver is excluded from the circulation, the immediate fall in blood pressure is prevented.[21] If this pooling is the result of neurovascular or neurohumoral effects originating in the brain or spinal cord, removal or destruction of these parts or their pathways should prevent fall of arterial blood pressure, rise of portal vein pressure, and associated pathologic changes after endotoxin. However, the characteristic changes were obtained in the chordotomized, vagotomized dog, and in the spinal cat. Moreover, the production of shock in the decapitated dog clearly negates the thesis that neurohumoral mechanisms arising in the brain are essential.

The manifestations of shock due to endotoxin are in many ways closely allied to those of anaphylactic shock,[22] and it is of considerable interest that the earliest workers studying anaphylaxis also proposed the theory that shock was due to profound chemical effects on the central nervous system. In 1909, Anderson and Rosenau[23] compared the manifestations of anaphylaxis and endotoxin shock and also proposed that both were the result of overwhelming toxic effects on the central nervous system. In refutation of these arguments, Pearce and Eisenbrey[24] reported that decapitation and mechanical destruction of the spinal cord did not protect against anaphylactic shock.

The intact nervous system may actually afford some protection against the progression of shock produced by the injection of endotoxin. In the present studies, the shock was more rapidly fatal in dogs after chordotomy and especially after the decapitation. However, this may well have been a more general effect of surgical trauma and not necessarily the result of the neurological deficit which was produced by the operation.

The protective effects of anesthesia and sedatives in both anaphylactic shock and shock due to endotoxin administration have been firmly established.[25, 26] Abernathy and Halberg[26] have recently demonstrated that chlorpromazine offers striking protection from the lethal effects of endotoxin in mice and they postulated that this involved a neurogenic mechanism. On the basis of such data it appears most reasonable to assume that the central nervous system is an important determinant in the progression of shock. Phemister and associates[27] have shown increased resistance to hemorrhagic shock following transection of the cervical spinal cord, and Wang and Overman[28] have been able to decrease resistance to hemorrhagic shock by strong sciatic nerve stimulation. Shock due to endotoxin, like shock caused by hemorrhage, need not be

traced to a primary injury of the central nervous system in order to establish that, once shock has been produced, nervous factors may be of great importance in its subsequent course.

SUMMARY AND CONCLUSIONS

Endotoxin from *Br. melitensis* and *E. coli* when injected intravenously into the dog causes characteristic symptoms, hemodynamic alterations principally manifested by arterial hypotension and transient portal hypertension, and typical morphologic changes. With use of the cross-circulation method of Penner and Klein in dogs, which has been presumed to separate the cephalic and the body circulations, shock and the classical pathologic changes were not limited to the animal whose brain was perfused with endotoxin. Arterial hypotension was not prevented by chordotomy, with and without vagotomy. A prompt fall in blood pressure and elevation of the portal vein pressure also occurred in the decapitated dog preparation. Anesthetized cats displayed a similar though more variable response to endotoxin than dogs. When the entire spinal cord in cats was destroyed by pithing, endotoxin still caused a profound secondary fall in arterial pressure, a small elevation of portal vein pressure, and characteristic morphologic changes. These investigations do not support the thesis that the initial manifestations of shock produced by endotoxin are the result of a direct action on the central nervous system. It is to be pointed out, however, that the central nervous system may have an important influence on the subsequent course and the lethality of shock after it has been precipitated.

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OCCURRENCE AND MODE OF ACTION OF ENDOGENOUS CIRCULATING ANTICOAGULANTS

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THE increasing evidence of physiologic anticoagulant(s) in normal blood has focused the attention of hematologists on the role of these blood clotting inhibitors in hemorrhagic diatheses. Various types of anticoagulants of high titer have been described in the circulating blood of some bleeding patients. An increase in antithrombin activity is not necessarily associated with bleeding and is not likely to be the sole cause for a hemorrhagic diathesis when bleeding is present. Other types of potent anticoagulants were found but are probably not involved in normal hemostasis (heparin and thromboplastin- or thromboplastin precursors–inhibitors). The presence of large amounts of heparin and heparin-like substances in the blood can be demonstrated during anaphylactic or peptone shock in animals[1, 2] and sometimes in severe hemorrhagic diathesis in man.[3] The diagnosis of hyperheparinemia is made by neutralization of the anticoagulant with protamine sulfate or toluidine blue and by the antithrombic and antithromboplastic properties of patients' blood. These cases are very rare.

The present study will be limited to the more usual and somewhat severe hemorrhagic diatheses caused by an inhibitor of the first phase of blood clotting, i.e., the formation of active thromboplastin.

Approximately 61 different cases of bleeding patients with a first phase anticoagulant in the peripheral blood have been carefully described in the past ten years (Table I, *4* to *54*). This group can be divided into five categories: circulating anticoagulants occurring, (1) in previously healthy males, 16 cases; (2) in patients suffering from hemophilia (classical hemophilia or antihemophilic globulin deficiency), 18 cases; plasma thromboplastin component deficiency (P.T.C., or Christmas disease), two cases; (3) in females following pregnancy (not later than 12 months after parturition), 16 cases; (4) in females where the bleeding tendency is not related to pregnancy, 8 cases; (5) in the newborn, 1 case.

In some instances the same case has been reported twice by a different group of authors.[18 and 19, 8 and 21, 32 and 33, 40 and 41] These patients are only reported once in Table I. It may be that the patient reported by Loveman[37] is the same as that described by Hewlett[40] and Heinle.[41]

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TABLE I. PUBLISHED CASES OF HEMORRHAGIC DIATHESES ASSOCIATED WITH THE PRESENCE OF A CIRCULATING ANTICOAGULANT IN THE PERIPHERAL BLOOD

CIRCULATING ANTICOAGULANT OCCURRING IN				
NONHEMOPHILIC MEN	HEMOPHILIC MEN	WOMEN, IN THE FIRST 12 MONTHS FOLLOWING PREGNANCY AND DELIVERY	WOMEN, NOT RELATED TO PREGNANCY	NEWBORN
	A. H. G. Deficiency			
1. Lozner and associates, 1940[4]	1. Lawrence and associates, 1946[18]	1. Evans and associates, 1931[31]	1. Joules and associates, 1938[47]	1. Frick, 1953[42]
2. Hall and associates, 1945[5]	2. Craddock and associates, 1947[19]	2. Franck, 1936[32] Fantl and associates, 1946[33]	2. Harrington and associates, 1950[48]	
3. Quick and associates, 1948[6]	3. Munro and associates, 1943[20]	3. Rosenthal and associates, 1937[34]	3. Pons and associates, 1952[49] and 1954[50]	
4. Conley and associates, 1948[7]	4. Lamy and associates, 1946[21]	4. Schultz and associates, 1945[35]	4. Nilsson and associates, 1953[51]	
5. Tzanck and associates, 1949[8]	5. Tzanck and associates, 1949[8]	5. Madison and associates, 1945[36]	5. Hardisty, 1954[52]	
6. Dieter and associates, 1949[9]	6. Conley and associates, 1948[7]	6. Madison and associates, 1945[36]	6. Evans, 1955[53]	
7. Singer and associates, 1950[10]	7. Singer and associates, 1950[10]	7. Loveman, 1945[37]	7. Evans, 1955[53]	
8. Deutsch, 1950[11]	8. Frommeyer and associates, 1950[22]	8. Chargaff and associates, 1946[38] Conley and associates, 1950[39]	8. Masure, 1956[54]	
9. Schmid, 1951[12]	9. Frommeyer and associates, 1950[22]	9. Hewlett and associates, 1949[40] first case		
10. Schmid, 1951[12]	10. Frommeyer and associates, 1950[22]	10. Hewlett and associates, 1949[40] second case, Heinle and associates, 1949[41]		
11. Schmid, 1951[12]	11. Frommeyer and associates, 1950[22]	11. Dreskin and associates, 1950[24]		
12. Collins and associates, 1952[13]	12. Frommeyer and associates, 1950[22]	12. Biggs and associates, 1953[42]		
13. Hougie, 1953[14]	13. Van Creveld and associates, 1953[23]	13. Frick, 1953[43]		
14. Quick and associates, 1954[15]	14. Dreskin and associates, 1950[24]	14. Beaumont, 1954[44]		
15. Goldstein, 1954[16]	15. Hougie, 1953[14]	15. O'Brien, 1954[45]		
16. Verstraete, 1955[17]	16. Hougie and associates, 1954[25]	16. Bruno and associates, 1954[46]		
	17. Verstraete and associates, 1954[26]			
	18. Frank and associates, 1954[27]			
	19. Baratta, 1954[28]			
	20. Quick and associates, 1954[15]			
	P.T.C. Deficiency			
	1. Soulier and associates, 1953[29]			
	2. Lewis and associates, 1953[30]			

Only anticoagulants inhibiting the formation of plasma thromboplastin are listed.

The anticoagulants in these patients may be called autoanticoagulants by analogy to the agglutinins. One case of transplacental transfer of an anticoagulant in the newborn has been reported.[43] A potent anticoagulant activity was also present in the blood of the mother at the time of parturition. The properties of the anticoagulant in the blood of the mother and the child were identical and the anticoagulant activity in the infant's blood disappeared progressively during the first 7 weeks.

This article concerns the mode of action and properties of the circulating anticoagulant complicating the hemorrhagic diathesis of a hemophilic patient

(antihemophilic globulin deficiency) and the endogenous anticoagulant occurring in an adult man who started to bleed profusely after 54 years of normal life. Our observations indicate that both anticoagulants are similar. These cases were previously reported in preliminary communications.[17, 26]

CASE REPORTS

CASE 1.—J. V. E., is a 17-year-old white schoolboy, with a history of excessive bleeding and prolonged bruising after minimal trauma, from early infancy. The family history is noncontributory. There is no consanguinity between the parents. The first bleeding tendency was noted when the umbilical cord atrophied and separated. The boy suffered from multiple ecchymoses when he started to walk. Epistaxis and gingivorrhagia occurred in this period and are still a frequent complaint. The diagnosis of hemophilia was made at this time in another hospital, based on a prolonged clotting time and normal platelet count, bleeding time, and capillary fragility test. At the age of 8, he had repeated hemarthrosis and a beginning ankylosis of the right knee. The first blood transfusion was given at the age of 11 and in the following years he was repeatedly transfused with whole fresh blood (500 ml. blood, ten times). The patient was admitted to the hospital three years ago with profuse bleeding after an attempted tooth extraction performed 48 hours before admission. The root was removed and the mucosa was sewn, closing the hole. The patient was transfused at the same time (400 ml. blood) and 2 ampules of human antihemophilic globulin (150 mg. of Cohn's fraction I) was injected (I. V.). He received 2,500 ml. of fresh blood and 1,500 mg. of Cohn's fraction I during the first six days. Since the slight hemostatic effect of the transfusions lasted for only an hour, the transfusions were stopped, although the red cell count was only 2,870,000 per cubic millimeter. Three days later the oozing subsided but the red cell count dropped to 2,080,000 per cubic millimeter. The first blood sample was sent to the physiopathologic laboratory 17 days after admission and a potent anticoagulant activity in the plasma and serum was detected. We were able to follow the progressive decrease of anticoagulant activity, which disappeared 7 months after it was discovered. It was then possible to prove by means of the thromboplastin generation test that the patient is a classical hemophiliac and has a 5 per cent antihemophilic globulin level. The patient received no further antihemophilic preparations and blood transfusions were usually avoided or restricted to packed cells.

CASE 2.—A. T., is a mason who first developed manifestations of a bleeding tendency in February, 1954, at the age of 54. He denied any abnormal bleeding previously although several tooth extractions were performed and he often injured himself while working. The family history is negative for blood dyscrasia and there is no consanguinity in previous generations.

The patient was in perfect health when one morning he noted a profuse ecchymosis on the dorsum of his left hand. Subsequently, he developed massive subcutaneous and intramuscular hemorrhages in the four extremities without precipitating trauma. The day he was admitted to the hospital, he was bleeding from the gums and had gross hematuria. Complete clinical, x-ray, and laboratory examinations failed to reveal any other cause of this severe anemia except the bleeding related to a circulating anticoagulant. The bone marrow presented the picture of hyperactive regeneration which is usually found in anemic patients. A potent anticoagulant interfering with the first phase of the blood clotting mechanism still persists in the plasma and serum of the patient.

METHODS

Collection of Blood.—Blood was collected by mixing 9 parts of blood with 1 part of 0.1 M trisodium citrate or, for some special experiments, with 0.1 M sodium oxalate. The blood was centrifuged for 6 minutes at 2,500 r.p.m. (low-spun plasma) or at 15,000 r.p.m. (high-spun plasma) and the plasma removed.

Clotting Time of Venous Blood.—Method of Lee and White.[55]

Recalcification Time (Howell Time).—Determination of the time interval between the addition of 0.1 ml. of 1/40 M $CaCl_2$ to 0.1 ml. plasma at 37° C. and the appearance of a clot.

Capillary Fragility Test.—Method of Rumpel-Leede,[56] modified by Wright.

Bleeding Time.—Method of Duke.[57]

Platelet Count.—Method of Van Goidsenhoven[58] using capillary blood and a urea solution.

One-Stage Prothrombin Time: One-tenth milliliter of citrated plasma was mixed with 0.1 ml. thromboplastin (acetone extract of human brain) and 0.1 ml. 1/40 M $CaCl_2$ at 37° C. and the clotting time recorded.

Two-Stage Prothrombin Time.—Method of Biggs and Macfarlane.[42]

Estimation of Prothrombin, Factor V and Factor VII.—The unknown citrated plasma is diluted 1:10 with Veronal buffer pH 7.35, ionic strength 0.154, and 0.1 ml. of this is added to 0:1 ml. aliquots of the substrate plasma. The clotting times are recorded after the addition of 0.1 ml. thromboplastin and 0.1 ml. 1/40 M $CaCl_2$. The substrates are respectively prothrombin, factor V- and factor VII-poor plasma prepared according to the methods of Owren.[59]

Prothrombin Consumption Test.—Blood was allowed to flow directly from the needle into dry glass tubes calibrated at 2 ml. All tubes were placed in a water bath at 37° C. At different time intervals 0.1 ml. serum was added to a fibrinogen solution and the clotting time recorded after addition of 0.2 ml. of a thromboplastin-$CaCl_2$ mixture. A 1.65 Gm. per cent in 0.85 per cent NaCl solution of bovine fraction I served as fibrinogen.

Partial Thromboplastin Test.—One-stage prothrombin times were done with various dilutions of tissue thromboplastin (acetone extract of human brain or thrombokinase Roche) or Russell viper venom. The thromboplastin suspensions were diluted with 0.85 per cent NaCl and the plasma with 0.85 per cent NaCl containing 0.38 per cent trisodium citrate.

Heparin Tolerance Test.—This test was performed with sodium heparin dilutions on citrated plasma as described previously.[60]

Thrombin Clotting Time.—Determination of the time interval between the addition of 0.1 ml. of various dilutions of thrombin solutions to 0.1 ml. citrated plasma and the appearance of a clot. Bovine thrombin was used (Thrombase Roussel).

Preparation of Platelet Suspension.—Blood was collected as described above and mixed with 1/10 0.1 M trisodium citrate or 1/19 volume of 1 per cent sequestrene solution (Na_2-ethylenediaminetetraacetic acid disodium salt). The glassware was coated with silicone. A differential centrifugation technique, as described by Biggs and Macfarlane,[42] was used.

Factor V and Antihemophilic Globulin (A.H.G.): Were prepared by the technique of Biggs and Macfarlane.[42]

Plasma Thromboplastin Component (P.T.C.).—According to the method of White, Aggeler, and Glendening.[62]

Thromboplastin Generation Test.—The method as described by Biggs and Douglas[61] was used. In this test the materials necessary for thromboplastin generation (platelets, antihemophilic globulin, factor V, and serum) are incubated together at 37° C. with $CaCl_2$. At one-minute intervals, the thromboplastin activity is determined by noting the time interval between the transfer of a subsample of the reacting mixture to recalcified high-spun plasma and the appearance of a firm clot. As this reactive mixture contains no prothrombin, the coagulation of the substrate plasma is considered to be dependent only on the thromboplastin developed in the mixture. A thromboplastin dilution curve may be constructed relating clotting times to thromboplastin concentration. The results of the thromboplastin generation test in this paper are expressed in per cent of maximal plasma thromboplastin yield developed by normal reactives. Although the conversion of clotting times to per

cent entails the risk of decreased accuracy in one's data, we consider this manner of expression necessary to compare results obtained on different days. Normal reactives were considered suitable for use only if the minimal clotting time was between 9 and 12 seconds and occurred within the third and seventh minutes. There is some confusion about the term "thromboplastin generation test" because the thromboplastin evaluated is not identical to what is generally called "thromboplastin." Even the originators of the method use the term "thromboplastin time" instead of "Quick time" (Biggs and Macfarlane[42]). The name "prothrombinase generation test" (terminology of Owren) would be more appropriate if one attributed the same properties to intrinsic (plasma) and extrinsic (tissue) thromboplastin. The activity of both intrinsic and extrinsic thromboplastin is similar to that of a platelet-antihemophilic globulin and P.T.C. complex.

RESULTS

Platelets.—The platelet count and agglutination were normal in both patients. The functional activity of the platelets was assessed by testing a platelet suspension from each patient for its ability to form plasma thromboplastin with factor V, antihemophilic globulin, and serum. In both instances the thromboplastin generation test was normal. As the clot retraction rate and the bleeding times were also normal, one may conclude that the functional activity of the platelets was adequate.

One-Stage Prothrombin Time (Human Brain Thromboplastin).—The results of the one-stage prothrombin time varied between 50 and 100 per cent of normal in Case 1; these differences were not related to the progressive decrease in the titer of the anticoagulant. The one-stage prothrombin time of Case 2 was always normal, thus excluding a deficiency of prothrombin or factors V and VII.

Antithrombin time was normal in the plasma of both patients.

Fibrinogen concentration in Case 1 was 365 mg. per cent plasma and was in Case 2 325 mg. per cent plasma.

Clotting time of venous blood was between 2 and 3 hours in Case 1, and about 4 hours in Case 2 (Lee and White method; normal less than 10 minutes). The recalcification time of citrated blood was always more than 360 seconds in both cases (normal plasma: 125 seconds ± 43 seconds).

Prothrombin Consumption Test.—Prothrombin consumption test (one-stage) was performed 4 and 24 hours after clotting. The results of this test revealed a marked defect in the consumption of prothrombin.

Test for Circulating Anticoagulants.—The presence of anticoagulant activity in a patient's blood, plasma, and serum may be demonstrated by the addition of the patients' plasma or serum in increasing proportions to normal blood or plasma. The clotting times of these mixtures become progressively longer. Fig. 1 demonstrates the anticoagulant activity of platelet-poor plasma. The recalcification times of these mixtures are recorded (37° C.).

The results of these tests demonstrate the presence of a significant anticoagulant activity in the plasma of both patients, and the decrease of the titer during the six months following its detection in Case 1. The presence of an anticoagulant activity may also be demonstrated by the impaired

prothrombin consumption in a mixture of normal blood (9/10) and patient's plasma (1/10). Table II shows the prothrombin consumption times of 1.8 ml. normal blood mixed immediately after withdrawal with 0.2 ml. plasma of Case 1. The patient's blood was previously treated with $BaSO_4$ (50 mg. per milliliter oxalated plasma) in order to adsorb prothrombin, factor VII, and P.T.C. We found in previous experiments that the anticoagulant is not adsorbed by inorganic salts [$BaSO_4$, $Al(OH)_3$, $BaCO_3$ . . .]. As can be seen in Table II the anticoagulant activity in Case 1 was most potent in May, 1953, and gradually dropped during the following months.

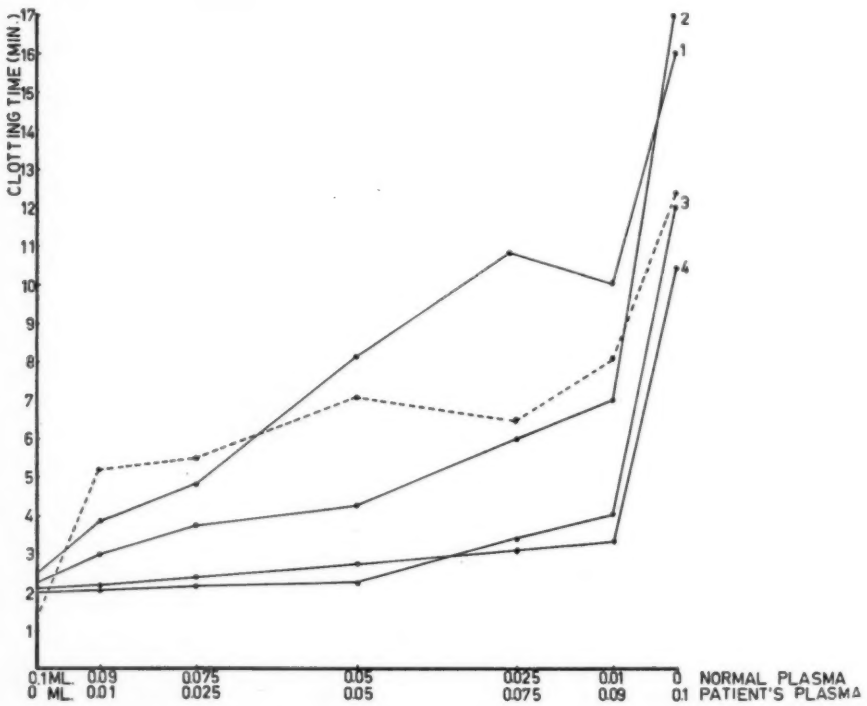

Fig. 1.—Recalcification times of mixtures of normal citrated plasma with citrated plasma of Patient 1 (continuous line) or Patient 2 (dotted line). Curve 1: plasma of Patient 1 drawn on 6/12/53; curve 2: 7/12/53; curve 3: 12/16/53; curve 4: 5/14/54.

The most sensitive method in our hands for detecting an anticoagulant in the first phase (thromboplastin formation) is the thromboplastin generation test.[17] If platelet-poor plasma of Patient 1 or 2 is used instead of normal high-spun plasma as the clotting substrate, normal generation of thromboplastin occurs. This indicates that the anticoagulant does not interfere with *formed* plasma thromboplastin and the generation and action of thrombin. On the other hand, the anticoagulant inhibits the formation of active plasma thromboplastin as will be demonstrated in the following experiments. All the reactives used in the thromboplastin generation test are prepared from normal blood and added to a tube containing 0.2 ml. $BaSO_4$-treated plasma or serum of Case 1 or 2. The test is subsequently performed in the usual way

TABLE II. INFLUENCE OF 0.2 ML., NORMAL AND 0.2 ML. PLASMA OF CASE 1 ON THE CLOTTING TIME AND PROTHROMBIN CONSUMPTION OF 2 ML. NORMAL BLOOD

	1.8 ml. normal blood + 0.2 ml. normal $BaSO_4$-treated plasma	1.8 ml. normal blood + 0.2 ml. $BaSO_4$-treated plasma of case 1		
		(5/12/53)	(8/12/53)	(12/18/53)
Clotting time	8'30"	20'	14'30"	10'
Prothrombin consumption time: time after clotting				
30 minutes		20.5"	24.5"	54.2"
1 hour	49"	20.3"	27.5"	54.4"
1½ hours	55.4"	22"	26.8"	60.3"
2 hours	52.9"	21.8"	22"	1'03"
3 hours	1'02"	22"	34"	1'15"
18 hours	2'03"	39"	2'35"	2'48"

The added normal and patient's blood was previously treated with $BaSO_4$ (50 mg./ml.).
Method: Clotting time: Lee-White technique; prothrombin consumption test: one-stage method with human brain thromboplastin and fibrinogen solution.

and the results for serial plasma dilutions with 0.85 per cent NaCl are shown in Fig. 2. The generation of active plasma thromboplastin is still grossly impaired at a 1:40 dilution.

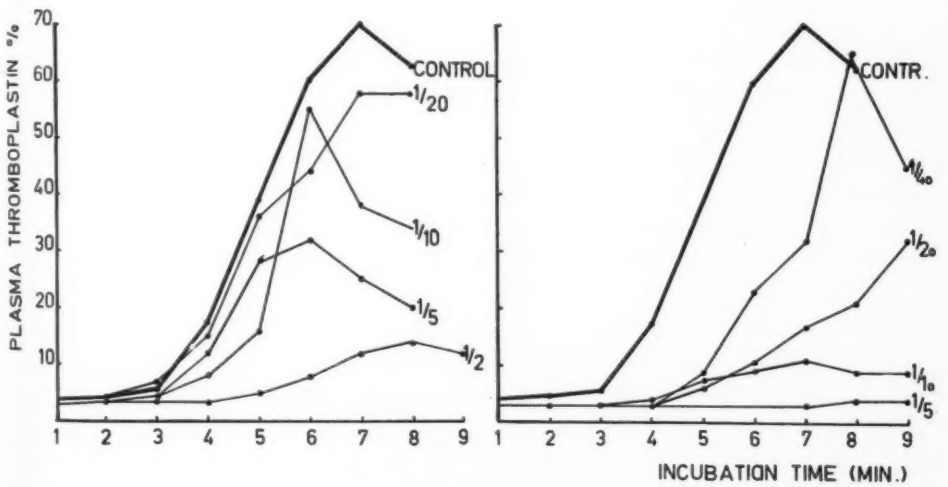

Fig. 2.—Generation of plasma thromboplastin by normal reactives (platelets, factor V, antihemophilic globulin, serum) in the presence of plasma of Patient 1 (left side of the figure) or Patient 2 (right side of the figure). The different reactives were added to the previous with $BaSO_4$-absorbed plasma of both patients. Dilutions of the absorbed plasma of the patients with 0.85 per cent NaCl.

Interference of the Anticoagulant in the Earlier Stages of Plasma Thromboplastin Formation.—Biggs, Douglas, and Macfarlane[63] have found that antihemophilic globulin, calcium, and P.T.C. react together to form an intermediate product in the earlier stages of thromboplastin formation. The following experiments were performed in order to evaluate the activity of the anticoagulants on this intermediate product. A method similar to the one described by Biggs and associates[64] was used. The antihemophilic globulin

TABLE III. INFLUENCE OF THE PREINCUBATION OF ANTIHEMOPHILIC GLOBULIN (A.H.G.), SERUM, AND $CaCl_2$ FOR 10 MINUTES AT 37° C. ON THE SPEED OF THE GENERATION OF THE PLASMA THROMBOPLASTIN

TIMES OF SUB-SAMPLING (MIN.)	REACTING MIXTURES			
	PLATELETS FACTOR V A.H.G. SERUM	PREINCUBATION (10′) OF A.H.G.; SERUM AND $CaCl_2$. SUBSEQ. ADDITION OF PLATELETS AND FACTOR V.	PREINCUBATION (10′) OF A.H.G., SERUM, $CaCl_2$ AND SERUM CASE 2. SUBSEQ. ADDITION OF PLATELETS AND FACTOR V.	PREINCUBATION (10′) OF A.H.G., SERUM, AND $CaCl_2$. SUBSEQ. ADDITION OF PLATELETS, FACTOR V, AND SERUM CASE 2.
	(%)	(%)	(%)	(%)
1	4	19	3	90
2	12	140	3	120
3	120	120	3	120
4	110	110	3	110
5	130	110	3	90
6	130	100	3	110
7	120	100	3.2	80

The anticoagulant present in the serum of Case 2 inhibits the generation of normal thromboplastin only when added before the preincubation of antihemophilic globulin, serum, and $CaCl_2$.

Method: The reacting mixture was tested for its thromboplastin activity by adding subsamples to platelet-poor plasma. The results are expressed in per cent of the normal thromboplastin activity.

and serum (source of P.T.C.) are preincubated in the presence of 1/40 M $CaCl_2$ at 37° C. for 3 minutes and the system then completed by the addition of platelets and factor V. It can be seen in Table III that preincubation is followed by a more rapid generation of thromboplastin. If a small quantity of serum from Patient 1 or 2 is added to the normal antihemophilic globulin fraction and normal serum before they are incubated, very little thromboplastin is formed. If the same or larger quantities of serum of Patient 1 or 2 are added to the antihemophilic globulin-calcium-serum mixture *after* their preincubation there is no influence on the next steps of the thromboplastin formation and the results of the tests are normal (Table III). The anticoagulant in both cases is therefore directed against the formation of the antihemophilic globulin-calcium-P.T.C. product. The next step in these investigations was to find out if the anticoagulant activity was directed against the antihemophilic globulin, the P.T.C., or both. The antihemophilic globulin preparation used was fraction I of bovine plasma which contains fibrinogen also, but is practically devoid of P.T.C. activity. Mixtures of fraction I with serum of the two patients results in a neutralization of their anticoagulant activity (Table IV). A P.T.C. preparation was obtained from normal serum according to the method of White, Aggeler, and Glendening.[62] Although a potent P.T.C. activity was obtained, it did not affect the anticoagulant activity in the serum of either case. This seems to indicate that the anticoagulant found in both of our patients acts against the antihemophilic globulin and not against the P.T.C.

The neutralization of the anticoagulant by fraction I occurs only in the presence of calcium ions. This need for calcium was already indicated by the failure of fraction I to neutralize the citrated or oxalated plasmas of both patients.

TABLE IV. INFLUENCE OF THE ANTICOAGULANT, PRESENT IN THE SERUM OF CASE 2, ON THE GENERATION OF PLASMA THROMBOPLASTIN WITH USE OF NORMAL REACTIVES (PLATELETS, FACTOR V, ANTIHEMOPHILIC GLOBULIN, AND SERUM)

REACTING MIXTURE PREPARED FROM NORMAL BLOOD	ADDITION TO REACTING MIXTURE	PLASMA THROMBOPLASTIN IN % OF NORMAL, GENERATED AFTER INCUBATION OF THE REACTIVES FOR						
		1 MIN.	2 MIN.	3 MIN.	4 MIN.	5 MIN.	6 MIN.	7 MIN.
- platelets - Factor V - antihemoph. globulin - serum	0.2 ml. 0.85 per cent NaCl	4	12	110	120	130	130	120
	0.2 ml. serum Patient 2, diluted 1:10 with 0.85 per cent NaCl	3	12	16	13.5	11	9	9
	0.2 ml. of a mixture of 0.5 ml. serum Patient 2, and 10 mg. of Cohn's fraction I. This mixture is diluted 1:10 with 0.85 per cent NaCl	5	70	90	110	120	120	120
	0.2 ml. of a mixture of 0.5 ml. citrated serum of Patient 2 and 10 mg. of Cohn's fraction I. This mixture is diluted 1:10 with 0.85 per cent NaCl	3	3	11	13.5	13.5	17	15
	0.2 ml. of a mixture of 0.5 ml. calcium-free dialyzed serum of Patient 1, and 10 mg. of Cohn's fraction I. This mixture is diluted 1:10 with 0.85 per cent NaCl	3	3	3	3	3	5	5

The anticoagulant activity is neutralized by Cohn's fraction I but only in the presence of calciumions.

Method: The reacting mixture was tested for its thromboplastin activity by the addition of sub-samples to platelet-poor plasma. The results are expressed in per cent of normal thromboplastin activity.

In the experiments summarized in Table IV, the influence of a small quantity of Cohn's fraction I on the anticoagulant present in the serum of Patient 1 is studied. As in previous tests, the anticoagulant activity is measured by adding 0.2 ml. serum diluted 1:10 with 0.85 per cent NaCl to normal reactives needed for the thromboplastin generation test (platelets, factor V, antihemophilic globulin, serum). When 10 mg. of Cohn's fraction I are added to 0.5 ml. serum of Patient 1, no inhibitory effect can be found any longer in the 1:10 dilution of this serum. Repeating the same experiments with citrated serum (1 ml. 0.1 M trisodium citrate for 9 ml. serum) or with calcium-free dialyzed serum of Patient 1, no neutralization of the anticoagulant activity with fraction I occurred. These experiments show that the anticoagulant of citrated serum or calcium-free dialyzed serum cannot be neutralized by fraction I (Table IV). Therefore, neutralization of antihemophilic globulin seems to occur by means of a calcium linkage.

These results only appear to contradict the data of Biggs and Macfarlane.[42] When they mixed the plasma of a patient having a circulating anticoagulant with antihemophilic factor, there was no correction of the clotting defect. The antihemophilic factor used, however, was not Cohn's fraction I, but the fraction precipitated from plasma by 33 per cent saturation with ammonium sulfate.

Some Properties of Circulating Anticoagulants.—As the mode of action of the anticoagulants present in the blood of hemophilic and nonhemophilic men was identical, it is of interest to compare some of their physiochemical properties.

The titer of anticoagulant activity in the blood or plasma of both patients is higher than in the respective sera. This indicates that part of the anticoagulant is consumed during blood clotting. The anticoagulant is stable on storage at –10° C. for at least two and one-half years but is relatively thermolabile. It resists heating at 56° C. for 40 minutes and at 60° C. for 10 minutes,

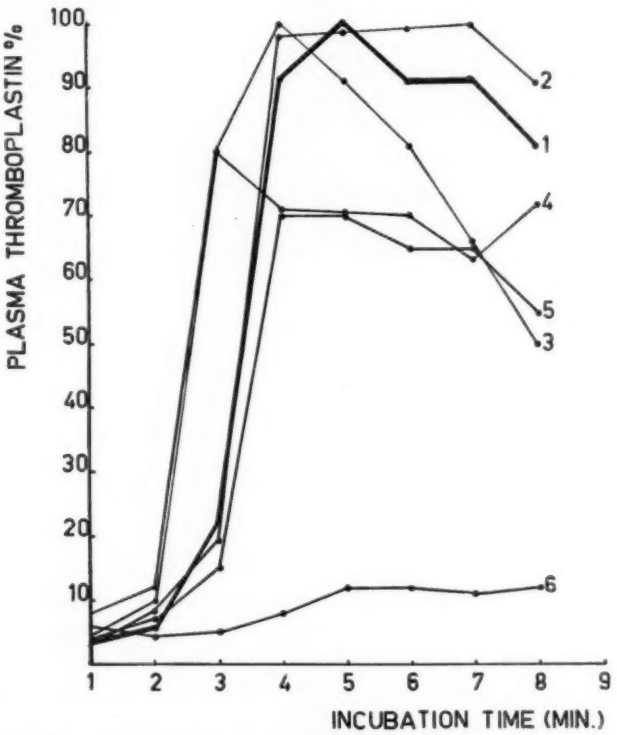

Fig. 3.—Influence of different serum protein fractions of Case 2 on the thromboplastin generation of normal reactives (platelets, factor V, antihemophilic globulin, serum). The proteins were separated by means of zone-electrophoresis and eluted with a Michaelisbuffer. Curve 1: curve of normal generation of plasma thromboplastin in the presence of 0.2 ml. Michaelisbuffer; thromboplasitn generated with normal reactives in the presence of 0.2 ml. of the albumin fraction (curve 2); of 0.2 ml. of the alpha$_1$ globulin fraction (curve 3); of 0.2 ml. of the alpha$_2$ globulin fraction (curve 4); of 0.2 ml. of the beta globulin fraction (curve 5); and 0.2 ml. of gamma globulin fraction (curve 6).

but the anticoagulant activity partially disappears after heating of the plasma to 70° C. for 10 minutes.[17] As previously stated, the anticoagulant is not adsorbed by inorganic salts ($BaSO_4$, $Al(OH)_3$) and is not dialyzable. We found that the proteins of the serum separated by zone electrophoresis have a normal pattern. The anticoagulant activity is present chiefly in the elution of the γ-globulin (Fig. 3). The different proteins strips were eluted with Michaelis buffer at pH 7.35.

We have previously shown that the one-stage prothrombin time was normal in Patient 2. In testing serial dilutions of tissue thromboplastin

(acetone-dried human brain or an animal lung preparation) against progressive dilutions of plasma, we were unable to find a difference between the normal plasma and the plasma of Patient 2. The same experiments were repeated with tissue thromboplastin heated at 70° C. for 3 minutes (Table V). The plasma containing the anticoagulant gives much longer clotting times with dilutions of heated thromboplastin than the control plasma.

TABLE V. INFLUENCE OF THE ANTICOAGULANT PRESENT IN THE PLASMA OF PATIENT 2 ON HEATED TISSUE THROMBOPLASTIN (ACETONE HUMAN BRAIN EXTRACT)

	TISSUE THROMBOPLASTIN (ACETONE HUMAN BRAIN EXTRACT) HEATED DURING 3 MIN. AT 70° C.				CONTROL 0.85 PER CENT NaCl
		DILUTED WITH 0.85 PER CENT NaCl			
	UNDILUTED	1:10	1:100	1:1,000	
Normal plasma:					
undiluted	20.5″	32.5″	44.5″	1.20″	2.43″
diluted with 0.85 per cent NaCl					
1:2	25.3″	37″	42″	1′20.5″	2′47″
1:4	39″	45″	48″	1′39″	3′18″
1:8	1′08″	—	1′06″	2′45″	5′54″
1:16	1′43″	—	1′15″	2′50″	7′32″
Plasma Patient 2:					
undiluted	21.1″	35″	1′10″	2′17″	7′33″
diluted with 0.85 per cent NaCl					
1:2	24.9″	38.5″	1′16″	2′17″	10′19″
1:4	31.5″	46.6″	1′22″	2′28″	11′45″
1:8	58.5″	52.7″	1′38″	2′46″	15′45″
1:16	53.7″	1′08″	1′42″	2′57″	17′45″

Method: 0.1 ml. plasma + 0.1 ml. tissue thromboplastin + 0.1 ml. $CaCl_2$ (37°C.).

It is known that tissue thromboplastins are phosphatideprotein complexes. An inactive protein can be separated from a slow clotting promoting lipoid fraction in lung thromboplastins. The mixture of both components restores the original activity of the lung extract.[65] Heating at 70° C. destroys the protein component of the tissue thromboplastin and makes it sensitive to the action of the circulating anticoagulants of both patients. Similar experiments were performed with Russell viper venom. The anticoagulant partly neutralizes the unheated thromboplastic agent; this effect is much more pronounced after heating of the Russell viper venom at 70° C. for 3 minutes.

Precipitin Test.—In order to investigate the presence or absence of antibodies against antihemophilic globulin, we used the ring method as described by Frommeyer, Epstein, and Taylor.[22] The antigens were (a) the fraction I of Cohn (Poviet, Amsterdam), (b) an antihemophilic globulin prepared by precipitation from normal plasma by 33 per cent saturation with ammonium sulfate, (c) normal citrated plasma, and (d) $Al(OH)_3$-adsorbed normal plasma. The control experiments were done with inert A.H.G. obtained by heating $Al(OH)_3$-treated plasma or fraction I at 56° C. for 15 minutes.

The absence of thrombin in the serum studied was tested by the addition of serum to $BaSO_4$-treated normal plasma or fibrinogen. The precipitin titer varied between 128 and 1,024 according to the antigens used. The test was

negative with the inert plasma and fraction I for all the sera. No precipitins were demonstrable once the circulating anticoagulant disappeared from the blood of Patient 1.

Therapeutic Trials in Cases With Circulating Anticoagulants.—In Case 1, the A.H.G.-deficient boy with a circulating anticoagulant, no treatment against this complication had been tried. Packed red cells were given in order to correct the anemia. As previously reported, anticoagulant activity in the blood disappeared progressively during the first six months.

Case 2 is the 54-year-old patient who never had a bleeding tendency previously but who developed a sudden severe hemorrhagic diathesis associated with the finding of a strong anticoagulant in the peripheral blood. The titer of the anticoagulant activity of his blood could not be modified by the I.V. administration of protamine sulfate (100 mg. of a 5 per cent solution), nor by the daily intake of 300 mg. toluidine blue for 25 consecutive days. These results are not surprising considering that the anticoagulant activity could not be neutralized in vitro by antiheparin agents.

One report of a successful treatment of an A.H.G.-deficient man whose disease was complicated by a circulating anticoagulant has been published.[23] Case 2 was given 100 mg. of hydrocortisone daily for 4 weeks and subsequently 40 I.U. of slow-acting ACTH Wilson daily for 24 days. No interpretable changes in the titer of the anticoagulant could be demonstrated. A trial with testosterone therapy (200 mg. of a depot preparation) every 3 weeks for 2 months had no effect on the hemorrhagic diathesis. The patient is still alive, 2 years after the onset of his bleeding disease.

DISCUSSION

These investigations show that the properties and mode of action of an endogenous circulating anticoagulant complicating a classical hemophilia (A.H.G. deficiency), or occurring in a nonhemophilic man are the same, or at least have similar properties.

These anticoagulants interfere only in the early stages of the first phase of the blood clotting mechanism. Formed plasma thromboplastin is not neutralized by the anticoagulant. The formation of an intermediate product between antihemophilic globulin, calcium, and P.T.C. is prevented; the anticoagulant activity is without influence once these clotting factors are linked. These results are in agreement with the work of Hougie and Fearnley.[25] In subsequent experiments we were able to show that the anticoagulant found in our cases acts on the antihemophilic globulin and not on the P.T.C. The anticoagulant activity can be exhausted by fraction I of Cohn but this occurs only in the presence of calcium. This reaction is reversible and can tentatively be schematicized:

(A.H.G. – Ca – anticoagulant) (P.T.C.) + fraction I and Ca ⇄
(A.H.G. – Ca – P.T.C.) (fraction I – Ca – anticoagulant)

The activity of the platelet suspension and factor V preparation of both patients was normal in its ability to form plasma thromboplastin.

Both circulating anticoagulants are relatively heat stable. Their activity remains constant at least two years when the plasma or serum is frozen at –10° C. These anticoagulants are nondialyzable, not adsorbed by inorganic salts, and not neutralized by protamine sulfate or toluidine blue in vivo or in vitro. Heated and diluted tissue thromboplastins are partly inactivated by the anticoagulants, indicating that it is the protein component of these thromboplastic agents which neutralizes the anticoagulant. Circulating anticoagulants have been reported in hemophiliacs type B (Christmas disease, P.T.C. deficiency).[29, 30] It would be very interesting to study the action of this anticoagulant against the Christmas factor and the antihemophilic globulin separately; until now such investigations have not been reported.

Anticoagulant activity was chiefly located in the gamma globulin fraction of the plasma and serum in our two cases. A positive precipitin test with various antihemophilic globulin preparations was also obtained with the serum of both patients. The circulating anticoagulants described by some other authors were found to be in the group of the beta and gamma globulins, with the maximum activity in the gamma globulins.[7, 9, 10, 14, 18-21, 23, 25, 51, 54]

TABLE VI. RESULTS OF THE PRECIPITIN TEST OBTAINED WITH THE SERUM OF PATIENTS HAVING A CIRCULATING ANTICOAGULANT AS REPORTED IN THE LITERATURE, INCLUDING AUTHORS' CASE

RESULTS OF THE PRECIPITIN TEST OBTAINED WITH THE SERUM OF PATIENTS HAVING A CIRCULATING ANTICOAGULANT		
Positive precipitin test:		
	hemophilic men:	2 cases[19]
		5 cases[22]
		1 case[25]
		1 case[17]
	nonhemophilic men:	
		1 case[25]
		1 case[26]
Negative precipitin test:		
	hemophilic men:	1 case[23]
		1 case[24]
	nonhemophilic men:	
		1 case[7]
		1 case[16]
	in females:	1 case[24]
		1 case[51]
		1 case[52]
		1 case[54]
Inconclusive results of the precipitin test:		
	hemophilic men:	1 case[10]
	in females:	1 case[42]
First considered positive, later retracted:		
	hemophilic man:	1 case[20, 22]

A positive precipitin test to antihemophilic globulin has been described in 11 cases of circulating anticoagulants; in 6 the report was negative and in 2 inconclusive (Table VI).

The fact that only some of the authors were able to find a positive precipitin test is most probably due to technical reasons. If the test is performed on blood obtained during the first 7 days after a plasma transfusion (or Cohn's fraction I) the patient may be in a negative antibody phase. It is well known that by giving antigen to a sensitized animal it is possible to reduce tem-

porarily the antibody titer. Frommeyer and associates[22] have demonstrated the decrease of precipitin titer against antihemophilic globulin in similar conditions in man. A sufficient concentration of antihemophilic globulin in the antigen seems also to be important in the performing of the test. The specificity is limited to the purity of the antigen, and the value of the test depends on the absence of prothrombin or free thrombin in the tested serum. As stated by Singer and associates,[10] negative results must be considered inconclusive, since the antigen is usually neither purified nor concentrated.

The fact that the anticoagulant is present in the gamma globulin fraction and that in several cases a positive precipitin test was found has given some weight to the theory that circulating anticoagulants are antibodies. This is based on the clinical observations that circulating anticoagulants could only be demonstrated in hemophiliacs who received several blood transfusions or, especially, antihemophilic globulin preparations (Cohn's fraction I). Frommeyer, Epstein, and Taylor[22] reported this finding in 5 patients out of a series of 16 cases of A.H.G. deficiency treated with Cohn's fraction I. This complication of hemophilia occurs more frequently in the United States than in Europe where antihemophilic globulin preparations are less often used. The frequent history of recent pregnancy in women with a circulating anticoagulant supports the possibility of an immunologic mechanism (in 20 of 25 cases the anticoagulant was detected within the twelfth month after parturition).

Conley,[7] Frick[67] and recently Lee[68] have studied 13 cases where a circulating anticoagulant appeared in the various conditions conventionally grouped under the term "collagen disease." It is well known that patients with lupus erythematosus disseminata develop early antibodies as evidenced by the false positive serology and positive Coombs test. It has been stressed that the anticoagulant in these cases, however, was active against formed tissue thromboplastin and therefore has a different mode of action from the anticoagulants described in this paper.

Little comment can be made with regard to therapy. Since the immunologic mechanism may be involved in the pathogenesis of circulating anticoagulants, cortisone and adrenocorticotropic hormone (ACTH) have been tried by different authors. The reports of two cases of hemophilia complicated by a circulating anticoagulant treated with these hormones were discouraging.[10, 14] Six nonhemophilic men with circulating anticoagulants[7, 13-16, 25, 66] and three women with the same disease[43, 50, 54] did not respond to ACTH or cortisone treatment. Large and prolonged doses of cortisone and ACTH were administered to Case 2 without a significant change in the titer of the anticoagulant. These results conflict with the data of Van Creveld and associates[23] who demonstrated a decrease in the titer of the anticoagulant in an A.H.G.-deficient man. Evans[53] also reported a decrease of the bleeding tendency in a woman having a circulating anticoagulant and who was treated with cortisone. Testosterone, protamine sulfate, and toluidine blue have been tried unsuccessfully in Patient 2. Since large transfusions (9 pints of fresh blood and two and one-half pints of fresh plasma) given during a period of 48 hours failed to control

the bleeding of a patient described by Hougie and Fearnley[25] it is advisable to avoid plasma or plasma fractions administration. Packed and washed red cells are helpful in treating anemia.

It should be mentioned that the bleeding tendency manifests itself in a similar way in patients with noncomplicated antihemophilic globulin deficiency, P.T.C. deficiency, in those cases where circulating anticoagulants occur in both conditions, in nonhemophilic males, or in females. There are no petechiae, but spontaneous bleeding may occur in deep tissues, or bleeding into the joints may occur following minor trauma.

SUMMARY

1. The occurrence of different types of circulating anticoagulants in hemorrhagic diatheses has been described. An anticoagulant acting in the first phase of the blood clotting (formation of thromboplastin) is the most common. Approximatively 60 cases of bleeding patients with such a first phase inhibitor in peripheral blood have been reported in the recent literature.

2. The mode of action and some physiochemical properties of the circulating anticoagulant complicating the bleeding tendency of a hemophilia type A patient and occurring in a nonhemophilic man were compared. Both first-phase inhibitors are present in the serum. They are stable for months when stored at –10° C., are relatively thermostable, not dialyzable, and are not adsorbed on $BaSO_4$ or $Al(OH)_3$. Their activity is not neutralized by protamine sulfate. The anticoagulant is chiefly present in the gamma fraction of plasma or serum globulins.

3. Both anticoagulants prevent plasma thromboplastin formation, but are inactive on plasma thromboplastin already formed. When antihemophilic globulin + Ca^{++} + P.T.C. are linked, the anticoagulant has no further influence on the subsequent stages of blood coagulation. The anticoagulant does not inactivate tissue thromboplastin, unless the latter is heated at 70° C. for 3 minutes.

4. The anticoagulant activity can be neutralized by Cohn's fraction I, but only in the presence of calcium ions.

5. The administration of protamine sulfate (P.O.), toluidine blue (P.O.), testosterone (I.M.), hydrocortisone (P.O.), or slow-acting ACTH has no influence on the anticoagulant activity found in the blood of a nonhemophilic man.

6. Some arguments suggesting an immunologic mechanism of development of the anticoagulant are presented.

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SOME EFFECTS OF ULTRASONIC ENERGY UPON BLOOD COAGULATION

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INTRODUCTION

METHODS previously employed for preparation of platelet extracts have included rapid alternate freezing and thawing, pulverization, exposure to hypotonic or hypertonic solutions, and a variety of other mechanical or physical procedures. None has been completely satisfactory, primarily because complete platelet lysis could not be accomplished. Attempts to find an efficient mode of platelet disruption, in order to prepare cell-free platelet extracts for study of their effect upon blood coagulation, led to the utilization of ultrasonic energy.

Wood and Loomis,[1] in an extensive study of the biologic effects of high frequency sound (ultrasound) waves, described rapid fragmentation of small living cells. Ultrasound has also been held accountable for molecular rearrangement of proteins, for initiation and acceleration of oxidations and other chemical reactions, and for effects resulting solely from the heat generated during the procedure.[2, 3] Sonic energy would be a valuable means of preparing platelet extracts only if it could accomplish rapid complete platelet breakdown with minimal chemical or physicochemical alteration of platelet constituents. Three studies of the effects of sonic energy on blood coagulation have been encountered. Burstein and Lewi[4] found that 10-minute exposure to 980 kilocycles per second, at 150 watts, rendered citrated plasma and fibrinogen incoagulable. Lenzi and Degli Esposti[5] reported uniform decrease in "Quick Prothrombin Time," and inconsistent decrease in "Howell Time" (recalcification time), of plasmas exposed to ultrasound of "medium intensity" for 5 minutes. Cole and associates[6] observed an average decrease in platelet count of approximately 80 per cent in platelet suspensions subjected to 10 kilocycles per second, at 200 watts, for 10 minutes, and found these platelet suspensions lacking in vasoconstrictor activity. However, they did not assay vasoconstrictor activity in platelet suspensions, similarly prepared except for exposure to ultrasound.

The present studies demonstrate that ultrasonic energy of 420 kilocycles per second, at 250 watts, can accomplish virtually complete platelet lysis in 60 seconds. Platelets lysed by ultrasound promoted greater acceleration of coagulation than did intact platelets, but were incapable of promoting clot retraction.

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None of the other platelet functions studied were altered by exposures for intervals up to 15 minutes. Sonic energy affects the coagulation of plasma; and data on the plasma defect induced by ultrasound are presented.

MATERIALS AND METHODS

1. *Preparation of Platelet and Plasma Samples.*—Platelet-rich plasma was obtained by slow centrifugation from normal and hemophilic bloods, rendered incoagulable by ethylenediaminetetracetate (EDTA) in a concentration of 0.01 per cent. Platelets were isolated by centrifugation of platelet-rich plasma at 20,000 g for 30 minutes at 4° C., and the supernatant plasma was used as platelet-poor plasma. The isolated platelets were thrice washed in, and the initial plasma volume reconstituted with cold imidazole-buffered isotonic saline at pH 7.25. Platelet counts of the final platelet suspensions, done directly in counting chambers, were 600,000 to 900,000 per cubic millimeter. All apparatus used for the collection of blood and preparation of platelets and plasma had been coated with silicone.

2. *Biologic Reagents.*—Lyophilized acetone-extracted rabbit brain thromboplastin (Bacto thromboplastin; Difco), and topical bovine thrombin (Thrombin, Topical; Parke-Davis), were used. One per cent bovine fibrinogen was prepared by the method of Ware and associates.[7] Crude prothrombin was prepared by adsorption of normal oxalated plasma with barium sulfate, and elution with sodium citrate.

3. *Methods Used in Evaluation of Coagulation.*—Recalcification time was determined on 0.1 ml. samples of plasma or plasma-platelet mixtures (made incoagulable with EDTA), by observation of the time of onset of fibrin formation at 37.5° C. after the addition of 0.2 ml. of 0.025 M calcium chloride. All plasma specimens were equilibrated with a gas mixture containing 5 per cent carbon dioxide and 95 per cent oxygen, before recalcification.

For determination of heparin neutralizing activity, blood was collected with chilled silicone-coated equipment, mixed gently with 2 mcg. of heparin (Heparin Sodium Injection; Lederle) per milliliter, and centrifuged at 4° C. to obtain platelet-poor plasma. Thereafter 0.2 ml. of heparinized plasma was transferred from the chilled siliconized container, to a glass clotting tube containing 0.1 ml. of saline or test substance, at 37.5° C. The time of onset of fibrin formation was noted as the end point. Controls were rechecked at the end of each experiment and found to remain constant.

Prothrombin conversion was measured by the two-stage prothrombin procedure of Ware and Seegers,[8] except in the experiment cited in "Results," section 2, b, where the modification used is described.

Labile factor was measured by the method of Quick[9]; serum prothrombin conversion accelerator (SPCA) activity by the method of Alexander[10]; prothrombin time by the one-stage method of Quick,[11] modified by the use of barium sulfate-adsorbed normal plasma as a diluent of the test plasma; and one-hour prothrombin consumption by the method of Quick.[21]

4. *Ultrasound.*—For ultrasonic treatment,* 2 to 5 ml. of test specimen was placed in Lusteroid tubes, or in plastic cylinders equipped with rubber diaphragms (both 28 mm. wide), and subjected to focused sound waves of 420 kc. per second at 250 watts for the desired period of treatment, which ranged from 30 seconds to 15 minutes. Since platelet lysis was usually complete in 1 minute, and no further alteration in platelet activity was ever noted after 3 minutes, exposure times of 1 to 3 minutes were usually used for platelet studies. The plasma coagulation defect was not consistently induced by exposure of less than 5 minutes.

*The Model BU2-4 hypersonic generator and Model BU-305A 400 hypersonic transducer, manufactured by the Brush Development Company, Cleveland, Ohio, were used. Electrical energy, supplied in the form of a high frequency signal by the generator, is converted into mechanical energy, in the form of high frequency sound waves by the piezoelectric ceramic bowl of the transducer, and transmitted through water to the test specimen. A continuous supply of cold water was maintained through coils surrounding the transducer, in an attempt to minimize heating. The temperature of test specimens, grossly checked with a standard laboratory thermometer, did not rise.

RESULTS

1. *Effects of Sonic Energy on Platelet Morphology.*—Examination of ultrasonically treated platelet suspensions by phase contrast microscopy usually revealed almost complete absence of intact platelets after 1 minute of exposure at 420 kc. per second. Specimens treated for only 30 seconds retained less than half of their initial platelets, most of which appeared as degenerated "balloon" forms. Five-minute exposure to sound waves at 10 kc. per second, generated by magnetostriction, resulted in loss of more than half of the platelets, but the audibility of the sound and the length of exposure required made the use of this frequency less satisfactory than the higher one.

TABLE I. EFFECTS OF PLATELETS ON THE RECALCIFICATION TIME OF PLATELET-POOR NORMAL PLASMA

9 PARTS PLATELET-POOR PLASMA PLUS 1 PART:	PLATELET COUNT (PER CU. MM.)	RECALCIFICATION TIME (SEC.)		NUMBER OF STUDIES
		AV.	RANGE	
1. Saline	less than 400	280	200-350	10
2. Platelet-rich plasma	60,000–90,000	147	128-175	7
3. Sonically treated platelet-rich plasma	60,000–90,000	99	85-110	5
4. Washed platelets	60,000–90,000	100	80-125	7
5. Sonically treated washed platelets	60,000–90,000	78	45-98	15
6. Supernatant of 4		165	——	1
7. Supernatant of 5		108	80-148	9
8. First wash of 5		114	98-138	3
9. Second wash of 5		130	125-138	3
10. Washed sediment of 5		109	95-123	7

2. *Effects of Ultrasonic Energy on Platelet Function.*—

a. *Recalcification time:* The over-all effect of platelets on coagulation was evaluated by the "recalcification time" (Table I). By this method, plasma containing 600,000 to 900,000 platelets per cubic millimeter (platelet-rich plasma), had an average recalcification time of 98 seconds (range 93 to 105). Test platelet preparations were added to fresh platelet-poor plasma in the ratio of 1:9. These plasma-platelet mixtures contained only 60,000 to 90,000 platelets (either intact or lysed) per cubic millimeter. The average recalcification time of plasma, with this concentration of intact, unwashed platelets (Table I, No. 2), was 147 seconds. The same concentration of unwashed platelets, sonically lysed in the plasma (No. 3), shortened the recalcification time to 99 seconds, and saline suspensions of sonically lysed, washed platelets (No. 5) shortened it even further, to 78 seconds. Platelets subjected only to the manipulation of several washings (No. 4) shortened the recalcification time to 100 seconds. The supernatants of successive saline washes of sonically treated platelet suspensions (Nos. 7 to 9) had diminishing accelerator effects, but the sediment (No. 10) retained significant activity after 3 washes.

b. *Prothrombin conversion:* Platelet accelerator activity was studied by means of a modification of a two-stage prothrombin procedure,[8] with use of

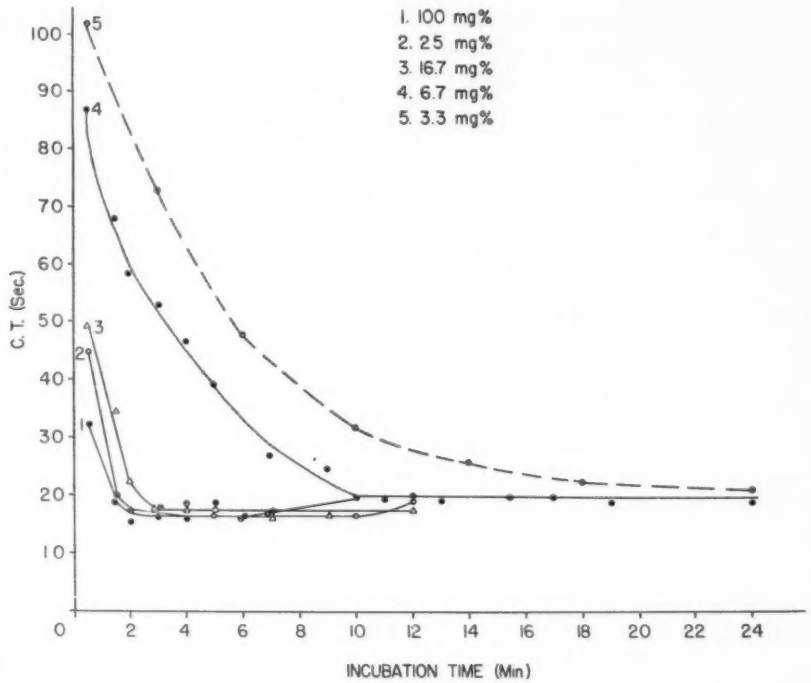

Fig. 1.—The effect of variation in thromboplastin concentration on the prothrombin conversion of normal plasma. The concentrations given refer to thromboplastin in the conversion mixture. Prothrombin conversion is not altered until the thromboplastin concentration is reduced to less than one-tenth the concentration generally used in the two-stage prothrombin procedure. (Note curves 1 and 4.)

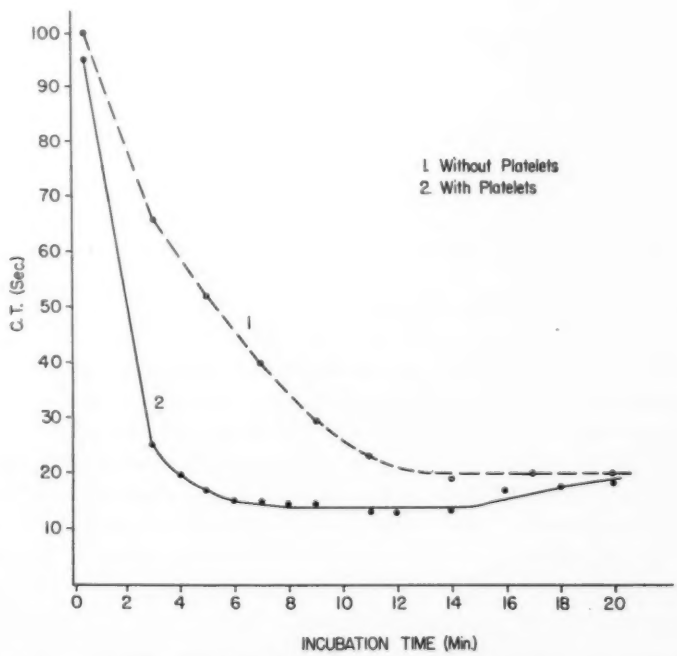

Fig. 2.—The effect of platelets (100,000 per cubic millimeter) on the prothrombin conversion of normal plasma in a thromboplastin-deficient system.

diluted normal plasma as the source of prothrombin. Fresh plasma, when incubated with an excess of rabbit brain thromboplastin, contains sufficient accelerators to give maximal conversion of prothrombin to thrombin, and no additional effect can be elicited by addition of platelets. Consequently, the effect of platelet preparations on prothrombin conversion was evaluated by their ability to compensate for a suboptimal concentration of thromboplastin. Fig. 1 illustrates an attempt to find a suitable suboptimal concentration of thromboplastin. Delayed prothrombin conversion was not evident until the concentration of thromboplastin in the conversion mixture was less than approximately 10 mg. per cent (curve 4). A 3.3 mg. per cent concentration (curve 5) was selected for these studies.

Fig. 2 illustrates the typical effect of platelets (100,000 per cubic millimeter of reaction mixture) on prothrombin conversion in the system containing 3.3 mg. per cent thromboplastin. The platelets were added after defibrination of the plasma with thrombin. An optimal range of platelet concentration seemed to exist, which compensated completely for the thromboplastin deficiency. Above or below this range, the reaction was slower. Generally, sonically treated and intact platelets had similar activity, but the optimal concentration for treated platelets (5,000 to 100,000 per cubic millimeter) tended to be lower than that for intact platelets (50,000 to 150,000 per cubic millimeter). Large excesses of intact or lysed platelets (more than 300,000 per cubic millimeter) were consistently detrimental to prothrombin conversion.

TABLE II. HEPARIN NEUTRALIZING EFFECT OF PLATELETS

HEPARINIZED (2 MCG./ML.) PLATELET-POOR PLASMA, PLUS:	CLOTTING TIME (SEC.) STUDY 1	STUDY 2	STUDY 3	AV.
1. Saline	780	720	840	780
2. Washed platelets*	100	115	130	115
3. Sonically treated washed platelets*	103	80	115	99
4. Supernatant of control platelets	320	—	—	
5. Supernatant of treated platelets	185	167	130	160
6. Sediment of treated platelets†	140	130	90	120

*Approximately 600,000 platelets per cubic millimeter.
†The sediment was washed 3 times and restored to its initial volume with saline.

c. *Antiheparin activity:* Heparin-neutralizing activity was evaluated by measuring the ability of platelets to counteract the anticoagulant action of 2 mcg. of heparin per millimeter of platelet-poor plasma (Table II). This activity was, perhaps, slightly augmented by sonic treatment of the platelets. Intact washed platelets and sonically treated washed platelets shortened the clotting time, at 37.5° C., from 780 seconds to 115 and 99 seconds, respectively. The washed sediment of treated platelets was more active than the supernatant.

d. *Vasoconstrictor activity:* Platelet vasoconstrictor activity was biologically assayed on strips of bovine carotid artery.[13]* Fig. 3 is a kymograph tracing of the effect of recalcified platelet-rich plasma on an arterial strip. Serum from sonically treated plasma elicited a more marked vasoconstrictor effect than did serum from the untreated control plasma.

*Assay of vasoconstrictor activity was carried out by Dr. Frederick S. Bigelow.

e. *Clot retraction:* Clot retraction was observed grossly. Sonically lysed platelets completely lost their ability to promote the retraction of clots of platelet-poor plasma.

f. *Hemophilic platelets:* Normal and hemophilic platelets were compared in all the studies, and no differences between them were discernible.

3. *Effects of Ultrasonic Energy on Other Phases of Coagulation.*—

a. *Thromboplastin:* Commercial rabbit brain thromboplastin, suspended in saline, was exposed to sound waves at 420 kc. per second for 1 to 15 minutes, and its residual potency was evaluated by means of the recalcification time, the heparin neutralization test, and the one- and two-stage prothrombin procedures (Table III and Fig. 4). Each of these tests demonstrated that sonic energy causes deterioration of this thromboplastin.

TABLE III. EFFECTS OF SONIC ENERGY ON THE ACTIVITY OF COMMERCIAL TISSUE THROMBOPLASTIN

COMPONENTS	RECALCIFICATION TIME* (SEC.)	HEPARIN CLOTTING TIME† (SEC.)	PROTHROMBIN TIME‡ (SEC.)
Saline	260	750	
Control thromboplastin	33	15	27.5
Sonically treated (15 min.) thromboplastin	50	30	55.0

*Nine volumes of platelet-poor plasma were mixed with one volume of thromboplastin (15 mg./ml.).

†Five-tenths milliliter of heparinized plasma was added to 0.1 ml. (3.75 mg.) of thromboplastin.

‡Plasma diluted 1:10 with fresh prothrombin-free plasma.

The curves in Fig. 4 represent prothrombin conversion in a system containing 100 mg. per cent thromboplastin. Curve 1 was obtained with untreated thromboplastin, and curves 2 to 4 with thromboplastin treated for 1, 5, and 15 minutes, respectively. Increasing the sonic exposure time resulted in both a decreasing rate of prothrombin conversion and a decreasing yield of thrombin.

b. *Plasma:* Normal plasma, with or without platelets, subjected to sonic energy for more than 3 minutes (in a few cases after 1 minute) manifested defective prothrombin conversion, partially lost its ability to correct the defects characteristic of aged and hemophilic plasmas when used in these tests, and acquired properties suggestive of anticoagulant activity. SPCA generation in

TABLE IV. EFFECTS OF SONIC ENERGY ON PLATELET-RICH PLASMA

COMPONENTS	RECALCIFICATION TIME (SEC.)	PROTHROMBIN TIME (SEC.)	SPCA (%)	THROMBIN TIME* (SEC.)
1. Normal plasma	95	14.6	166	20
2. Sonically treated (5 min.) normal plasma	285	24.5	160	20
3. Hemophilic plasma	205			
4. Hemophilic and normal plasma (1:1)	98			
5. Hemophilic and sonically treated normal plasma (1:1)	170			
6. Normal and stored plasma (1:9)		23.0		
7. Sonically treated normal and stored plasma (1:9)		60.0		

*Four-tenths milliliter thrombin plus 0.1 ml. plasma.

Fig. 3.—The effect of sonic energy on the vasoconstrictor activity of platelet-rich plasma. Left: serum from untreated recalcified oxalated plasma. Right: serum from sonically treated (3 minutes) recalcified oxalated plasma.

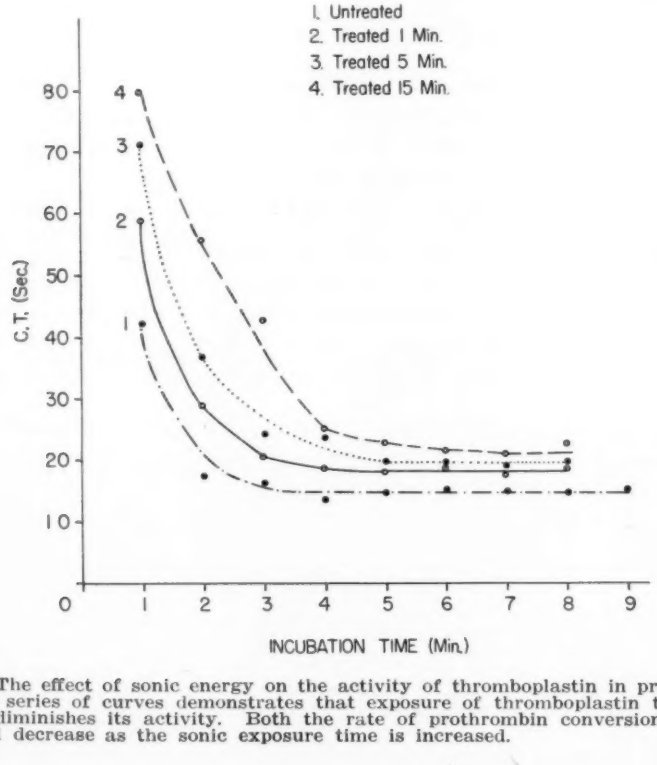

Fig. 4.—The effect of sonic energy on the activity of thromboplastin in prothrombin conversion. This series of curves demonstrates that exposure of thromboplastin to sonic energy progressively diminishes its activity. Both the rate of prothrombin conversion and the final thrombin yield decrease as the sonic exposure time is increased.

the treated plasma was normal. Table IV summarizes studies done on a sample of normal platelet-rich plasma, exposed to sonic energy for 5 minutes. The magnitude of the defect created by sonic energy varied considerably in the different samples tested, but the qualitative changes were uniform. The data presented in Table IV approximate the average findings.

TABLE V. EFFECT OF FRESH PLASMA ON THE DEFECT OF SONICALLY TREATED NORMAL PLATELET-RICH PLASMA

COMPONENTS	RECALCIFICATION TIME (SEC.)	PROTHROMBIN TIME (SEC.)
1. Control plasma	95	14.6*
2. Sonically treated (5 min.) plasma	285	24.5*
3. Control plasma and:		
a. Sonically treated plasma (1:9)	250	
b. Sonically treated plasma (1:1)	150	
c. Sonically treated plasma (9:1)	100	
4. Control plasma and:		
a. Prothrombin-free plasma (1:9)		26.2
b. Sonically treated prothrombin-free plasma (1:9)		51.0
c. Sonically treated prothrombin-free plasma (9:1)		28.0

*Undiluted plasma.

Fresh normal plasma, added in equal volume to sonically treated plasma, did not reduce the recalcification time to normal (Table V). It is also apparent, from the data in Table V, that the prolonged recalcification time of the sonically treated plasma occurred despite the presence of a high concentration of platelets during the exposure, and that the prolonged prothrombin time was obtained despite the subsequent addition of excess tissue thromboplastin. One part of sonically treated prothrombin-free plasma increased the prothrombin time of 9 parts of normal plasma (Table V, *4c*). This strongly suggests the presence of an anticoagulant. The possibility that all the plasma changes induced by ultrasound are partially due to an anticoagulant was not investigated further.

The defective prothrombin conversion of the sonically treated plasma was corrected by intact or by sonically treated platelets, when commercial tissue thromboplastin was also present, but it was not corrected by the presence of tissue thromboplastin alone (Fig. 5). The upper 3 curves (curve group 2) in Fig. 5 represent the results of 3 experiments on the serial clotting times of plasma, sonically treated for 5 minutes, and incubated with excess (100 mg. per cent) tissue thromboplastin. The progressive lengthening of clotting time was a consistent finding, and as yet is unexplained. However, as shown by curve 3, sonically treated platelets (80,000 per cubic millimeter in the reaction mixture) added to the sonically treated plasma, completely corrected the anomalous prothrombin conversion.

c. *Prothrombin, thrombin, fibrinogen, and serum:* Crude prothrombin and topical bovine thrombin were exposed to sonic energy and did not seem to be affected by it. Normal serum, exposed to sonic energy, lost some of its accelerator potency. Bovine fibrinogen was variably affected, its coagulability by thrombin being occasionally increased, occasionally decreased or abolished, but most frequently not affected at all. The erratic effects on fibrinogen were thought

to be due to uncontrollable variations in the intensity of the sonic output, and for all studies except those on fibrinogen itself, only plasmas which were tested and found to have intact fibrinogen were used.

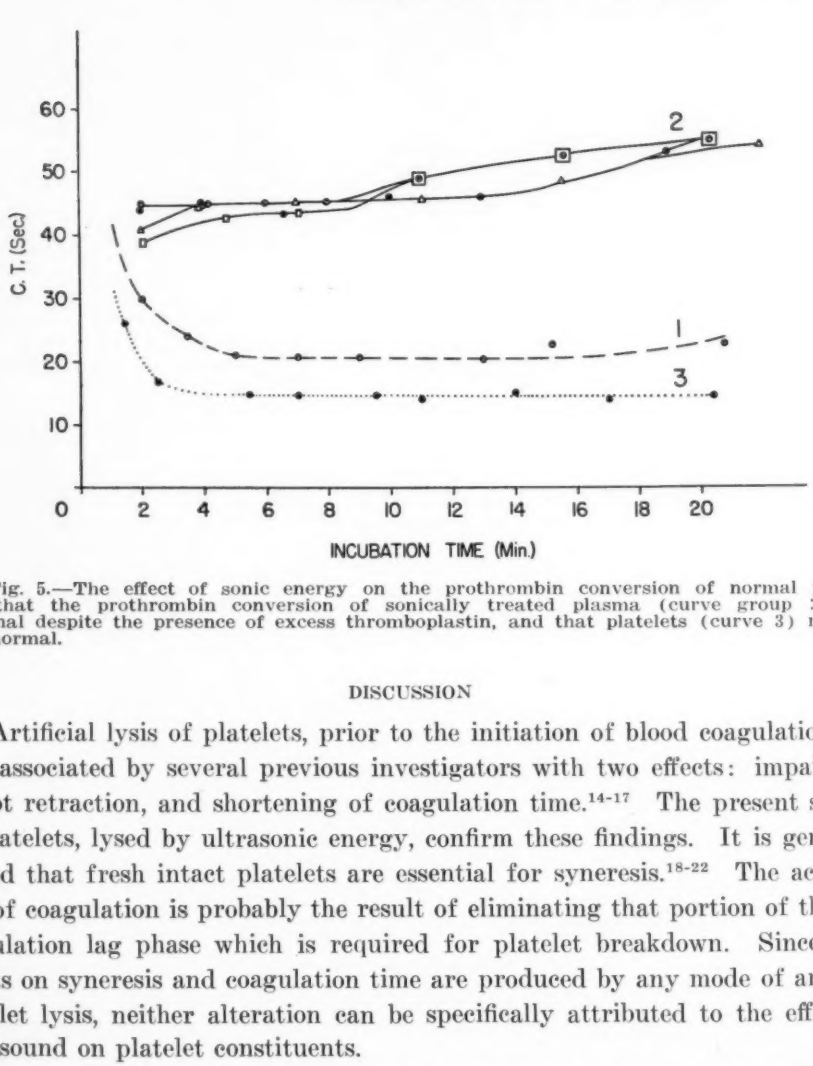

Fig. 5.—The effect of sonic energy on the prothrombin conversion of normal plasma. Note that the prothrombin conversion of sonically treated plasma (curve group 2) was abnormal despite the presence of excess thromboplastin, and that platelets (curve 3) restored it to normal.

DISCUSSION

Artificial lysis of platelets, prior to the initiation of blood coagulation, has been associated by several previous investigators with two effects: impairment of clot retraction, and shortening of coagulation time.[14-17] The present studies on platelets, lysed by ultrasonic energy, confirm these findings. It is generally agreed that fresh intact platelets are essential for syneresis.[18-22] The acceleration of coagulation is probably the result of eliminating that portion of the pre-coagulation lag phase which is required for platelet breakdown. Since these effects on syneresis and coagulation time are produced by any mode of artificial platelet lysis, neither alteration can be specifically attributed to the effects of ultrasound on platelet constituents.

The release of clot-promoting substance from platelets in the course of spontaneous blood coagulation was recognized early in the study of coagulation mechanisms,[23] but the nature of the substance or substances released is still under study. The platelet constituents released by sonic platelet lysis are not detectably altered by the treatment. The method is simple, rapid, and effective, and can be recommended as a means of preparing platelet extracts.

The activity of platelets is still attributed by some investigators to their content of a significant quantity of preformed thromboplastin.[24-26] Differences between active platelet components and commercial rabbit brain thromboplastin, were encountered in the present studies. Whereas sonic energy rapidly and progressively destroyed the activity of the rabbit brain thromboplastin, it had no adverse effect on the activities of platelet constituents. In addition, tissue thromboplastin was incapable of correcting the defective prothrombin conversion of sonically treated plasma, in the absence of platelets or platelet extracts. These observations support the probability that platelets do not owe their activity to the same substance that is active in lyophilized acetone-extracted rabbit brain.

The nature of the plasma defect created by sonic energy has not been completely studied, but the preliminary findings invite some speculation. Manifestations of the defect included: a prolonged recalcification time, a prolonged one-stage prothrombin time, anomalous prothrombin conversion by the two-stage prothrombin technique, the development of suggestive anticoagulant properties, and, more specifically, impairment of the ability of sonically treated plasma to correct the defects of aged (labile factor deficient) and hemophilic plasmas. All of these anomalies except the formation of an anticoagulant can be induced by heat. However, fibrinogen, which is destroyed when heated to 47° C., remained intact. This precludes the possibility that the effects of ultrasound on plasma were due solely to heat.

The presence of large numbers of platelets in the plasma during sonic treatment did not influence the nature of the defect. The addition of intact platelets to the treated plasma, or, equal mixture of the treated plasma with fresh normal plasma, shortened the recalcification time, but did not reduce it to normal. Only in the presence of both thromboplastin and platelets (both added after defibrination of the plasma) was normal prothrombin conversion, measured by the two-stage prothrombin procedure, restored. Platelets alone, and fresh plasma plus thromboplastin, were not tested by this method.

McClaughry and Seegers[27] postulate the existence of two classes of prothrombin conversion accelerators: thromboplastin, and the plasma and platelet accelerator globulins. They suggest that both must be present for effective prothrombin conversion. Deficiency of substances of either class can be made the limiting factor in thrombin evolution, and an excess of one class can compensate for a partial deficiency of the other, provided a critical minimal quantity of the deficient one is present. In the frame of this hypothesis, it would seem that sonic energy creates partial deficiencies of both types of accelerators, specifically, of antihemophilic substance in the thromboplastin class and of labile factor in the accelerator globulin class. Addition of critical quantities of both (a) thromboplastin and (b) either fresh plasma or platelets should be, and was found to be, necessary to correct this defect.

In the few completed studies on hemophilic blood, the following points were noteworthy. Hemophilic platelets behaved exactly as did normal platelets. Thus, physiologic concentrations of either normal or hemophilic platelets, thoroughly lysed by sonic energy, did not significantly accelerate the coagulation of

hemophilic plasma. Addition of highly concentrated platelet extracts* to hemophilic plasma did shorten the coagulation time, occasionally to normal, but did not significantly improve prothrombin consumption. These observations are in accord with the current belief that increased stability of the platelets is not a primary defect in hemophilia. They are compatible with the hypothesis that a large excess of platelet accelerator can partially compensate for a partial deficiency of antihemophilic globulin.

It is perhaps of interest that the recalcification time of hemophilic plasma was altered less than that of normal plasma by sonic treatment (Table VI).

TABLE VI. COMPARISON OF EFFECTS OF SONIC ENERGY ON THE RECALCIFICATION TIMES OF NORMAL AND OF HEMOPHILIC PLATELET-RICH PLASMAS

COMPONENTS	RECALCIFICATION TIME (SEC.)
1. Normal plasma	85
2. Sonically treated* normal plasma	285
3. Hemophilic plasma	185
4. Sonically treated* hemophilic plasma	255
5. Comp. 1 and comp. 2 (1:1)	150
6. Comp. 1 and comp. 3 (1:1)	100
7. Comp. 1 and comp. 4 (1:1)	200
8. Comp. 2 and comp. 3 (1:1)	195

*Treated for 5 minutes.

Antihemophilic globulin seems to be one of the substances injured by sonic energy. Since hemophilic plasma is initially deficient in this respect, the additional defect induced by sonic energy might be expected to be less impressive.

SUMMARY

1. High-frequency sound waves provided an effective means of rapidly lysing platelets without altering the accelerator, antiheparin, or vasoconstrictor properties of the platelet constituents which were released.

2. Suspensions of platelets, lysed by sonic energy, exhibited the same altered properties as did platelet suspensions lysed artificially by other techniques, namely: enhanced ability to accelerate plasma coagulation, and loss of the ability to promote syneresis.

3. No differences between normal and hemophilic platelets were observed either before or after exposure to sonic energy.

4. The activity of commercial rabbit brain thromboplastin was rapidly and progressively diminished by sonic energy. Since platelet activity was not impaired by comparable sonic treatment, it is suggested that this type of thromboplastin is not a significant component of platelets.

5. Sonic energy induced a coagulation defect in plasma, which seemed primarily, to be due to partial destruction of labile factor and of antihemophilic activity, and possibly to the formation of an anticoagulant.

6. Accelerator activity was diminished in sonically treated serum, but SPCA generation was unaffected. Sonic energy did not alter prothrombin or thrombin, and its effect on fibrinogen was variable.

*The extracts were prepared from 500 ml. of blood, fractionated by the method of Van Creveld and associates.[26, 28] In accord with the findings of these workers, the predominant antihemophilic activity resided in the water insoluble ("Factor 3") fraction. However, if the platelets were lysed by ultrasound prior to fractionation, the activity was transferred to the water-soluble fraction.

All ultrasonic procedures were carried out in the laboratories of the Ultrasonic Corporation of Cambridge, Massachusetts. I am deeply indebted to this corporation for so generously allowing the use of their equipment, and particularly to Mr. Bertram Harrison and Mr. Serge Wisotsky for their assistance.

I wish to thank Dr. William B. Castle and Dr. Frederick S. Bigelow for their valuable assistance with the preparation of this manuscript, and Mrs. Doris McAvoy for technical assistance.

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AMMONIA LEVELS IN TRANSFUSED BLOOD

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IN THE past several years a number of experimental and clinical observations have suggested that exogenous protein and other nitrogenous substances including ammonia may, under certain circumstances, be related to the syndrome of hepatic coma.[1-5]

Under normal conditions, the gastrointestinal tract and the kidney are the primary source of ammonia.[6] The level of ammonia in the portal blood is about four to five times that in the peripheral blood.[1] In derangement of hepatic function and/or portal-systemic shunts it is assumed that the ammonia content of peripheral blood may be elevated to critical levels. Under these conditions the addition of exogenous sources of ammonia might precipitate coma. In the cirrhotic patient, bleeding massively, blood replacement is one of the indicated supportive therapeutic measures. It is the purpose of this paper to submit data showing that there is a precipitous rise in the ammonia content of stored blood which is to be considered in the treatment of patients on the verge of hepatic insufficiency.

METHODS

The procedure used throughout this study was a combination of the interrupted diffusion method of Conway[7] and Russell's[8] phenol-hypochlorite procedure for the estimation of ammonia. Aliquots of whole blood were treated with an equal quantity of saturated potassium carbonate and the released ammonia allowed to diffuse into the center well of a Conway diffusion cell containing 3.5 ml. of 0.02 N H_2SO_4. Suitable blank and standard solutions were similarly treated.

At the end of a diffusion period of one hour at room temperature (25° C.) 3.0 ml. aliquots (or fractions thereof) were removed from the center well and analyzed by Russell's[8] colorimetric procedure. All values are the average of duplicate determinations and are expressed as ammonia nitrogen.

This procedure gave a satisfactory degree of precision. A series of twelve replicate analyses on stored blood resulted in a mean value of 208 mcg. per cent ammonia-N with a standard deviation of 11.0 mcg.

The bank blood studied throughout this investigation was obtained either by phlebotomy into 250 ml. ACD bottles (Baxter) or from the blood bank. Aliquots were removed by sterile syringe and needle after varying periods of storage at 4° C. In the study of the effect of glutamate, duplicate 250 ml. aliquots were obtained by phlebotomy and 5 ml. of 20 per cent monosodium glutamate was added to one sample. It should be

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noted at this point that no effort was made to complete analyses immediately after phlebotomy. Values given for zero days represent an interval of from 1 to 2 hours after the phlebotomy was completed.

RESULTS

Analysis of bank blood selected at random yielded a wide range of ammonia concentration (Table I).

TABLE I. "AMMONIA" LEVELS IN STORED BANK BLOOD SELECTED AT RANDOM

DAYS STORAGE	AMMONIA LEVEL (MCG. %)	DAYS STORAGE	AMMONIA LEVEL (MCG. %)
0	128	21	770
1	167	21	930
3	244	21	620
4	185	22	690
5	194	22	785
5	214	25	830
20	685		

In order to establish whether the increment in ammonia concentration is a gradual one that occurs throughout the storage period, serial determinations were carried out on blood collected and stored at 4° C. Fig. 1 indicates the values obtained and shows that there is a roughly linear increase in ammonia concentration with time, at least in the first 15 days of storage. After

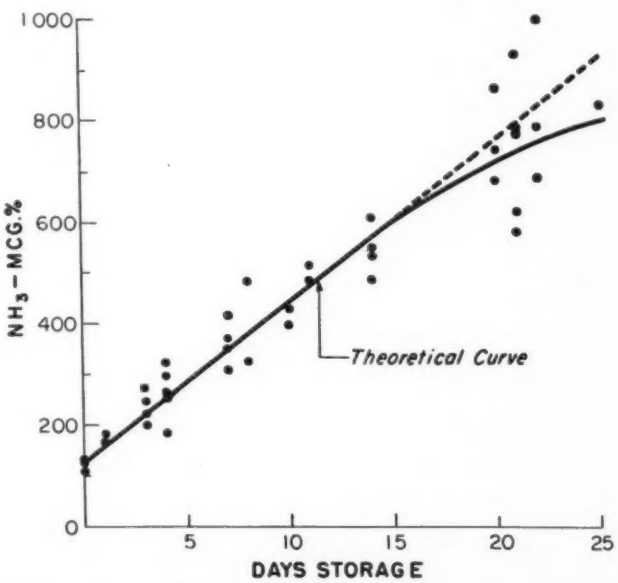

Fig. 1.—Distribution of "ammonia" levels in blood after varying periods of storage at 4° C.

this time, there seems to be a grouping of values below the extrapolated linear relationship suggestive of a depressed rate of ammonia formation. This depression in the rate of ammonia formation at higher initial levels of ammonia concentration becomes more apparent when both fresh and aged bank blood are subjected to additional storage at 37° C. to accelerate the rate of increase in ammonia concentration (Table II).

TABLE II. INCREASE OF "AMMONIA" CONTENT AFTER STORAGE OF BLOOD AT 37° C.

SAMPLE NO.	INITIAL LEVEL OF AMMONIA (MCG. %)	STORAGE AT 37° C.			
		2 HR. (MCG. %)	PERCENTAGE INCREASE	24 HR. (MCG. %)	PERCENTAGE INCREASE
1	278	322	16	—	—
2	147	172	17	1,000	480
3	834	924	10.5	1,348	46

After the addition of 5 ml. of 20 per cent glutamate to 250 ml. of blood, the rate of ammonia formation or appearance was depressed compared with a duplicate sample to which no glutamate was added. This depression was consistent in all stages of storage and amounted to a mean depression of 22 per cent (Table III).

TABLE III. EFFECT OF SODIUM GLUTAMATE ON THE INCREASE IN AMMONIA IN BANK BLOOD AFTER VARYING PERIODS OF STORAGE AT 4° C.

DAYS STORAGE	WHOLE BLOOD (NH_3–N MCG. %)	WITH 5 ML.* GLUTAMATE (NH_3–N MCG. %)	PER CENT DIFFERENCE
Sample 1			
0	108	88	–18.5
3	226	187	–17.3
4	298	223	–25.1
7	358	309	–13.7
10	430	342	–20.4
21	936	831	–11.3
			Mean = –17.7
Sample 2			
1	171	129	–24.5
4	326	232	–28.8
7	418	263	–37.0
18		635	
22	1,000	777	–23.0
			Mean = –28.3
Sample 3			
3	278	225	–19.9
7	374	291	–22.2
11	516	405	–21.1
14	552	459	–16.9
21	782	671	–14.1
			Mean = –18.8
			Combined mean = –22

*Values corrected for NH_3 content of 20 per cent glutamate.

It is possible that the markedly increased ammonia levels observed are due to the release of volatile base from proteins as a result of the analytical technique. This, however, is unlikely. Protein-free filtrates prepared from bank blood stored to raise the ammonia level were analyzed simultaneously with whole blood aliquots. Whole blood ammonia levels of 1.63 and 1.92 mg. per cent (corrected for differences in diffusion)[9] compared favorably with values of 1.58 and 2.14 mg. per cent for the respective protein-free filtrates.

Interference by glutamine is of considerable importance in the determination of normal blood ammonia concentrations. In our trials 500 mcg. of glutamine released approximately 1 mcg. of ammonia-N in 60 minutes at 25° C. This is in the range reported by others[18] and, with a blood glutamine level of from 10 to 20 mg. per cent, probably represents an error of roughly 20

to 40 mcg. per cent in our determination of ammonia in blood. The elevated "normal" values of several investigators[2, 10] as compared with the normal values of Conway[7] and McDermott[3, 16] can be explained, at least in part, on this basis. To explain the markedly elevated values reported herein on the basis of glutamine interference would necessitate an assumption of glutamine levels in the range of 500 mg. per cent. No evidence for interference of this magnitude was obtained when the results from 10-minute and 60-minute diffusion times were compared, supporting the belief that the volatile base exists as preformed ammonia in the bank blood.

DISCUSSION

It has been recognized for some time that ammonia appears to develop in the blood within minutes after shedding. On the basis of extrapolated values obtained from serial determinations of ammonia concentration after shedding, it has been suggested that the normal circulating ammonia level may be zero. Approximately 5 to 15 minutes after shedding, a plateau of ammonia concentration is reached and it has been stated that the subsequent ammonia level is fairly constant.[3, 11] This, however, has not been studied adequately.

In an early report on the content of ammonia in blood, Nash and Benedict[12] indicate that "there is a slight increase in the ammonia content after standing 30 minutes, but that further standing up to 3 or 4 hours causes no increase in the ammonia." Parnas and Klisiecki[13] make reference to values of 30 to 200 mcg. per cent after some storage and indicate that a storage of 24 hours (temperature not indicated) may result in an ammonia concentration of 1.5 mg. per cent. Since the advent of stored blood for transfusion purposes there has been, to our knowledge, no further evaluation of this problem.

The data presented above indicate that a marked increase in ammonia concentration does occur in blood during storage. Although only limited data are available, it would seem that this increase is roughly linear over most of the storage period. The addition of glutamate, suggested by the various reports on its use in vivo,[4, 16, 17] resulted in a mean reduction of 22 per cent in the formation of ammonia throughout the storage period. This limited effect indicates that further studies are desirable to measure its significance and mode of action. Although it has been suggested that the release of ammonia immediately after blood is shed is due to the deamination of adenosine,[11] this glutamate effect indicates that other mechanisms may be involved in the long-term release of ammonia.

It is clear, however, that the ammonia content of bank blood can be of clinical importance. Routine samples of blood selected for transfusion purposes may have an ammonia concentration of from 200 to 900 mcg. per cent or higher (Table I). When blood is used therapeutically in amounts approaching exchange transfusions, a considerable quantity of ammonia may be inadvertently administered. Since current therapy in impending coma involves, in part, the reduction of the formation of ammonia through dietary restriction, antibiotics, and the removal of stagnant blood from the gastrointestinal tract, the use of large quantities of aged blood must be re-evaluated.

The infusion of ammonia via large amounts of stored blood might well be expected to raise the circulating ammonia concentration, at least temporarily, with the possibility of subsequent neurological manifestations. Attempts at demonstrating this increase have been unsuccessful in one patient and further investigations await the availability of suitable cases.

Recent reports, however, indicate that a posttransfusion increment in ammonia level may not be demonstrable in venous blood. Bessman and his collaborators[14, 15] have presented data suggesting that muscle can remove approximately 40 per cent of the ammonia presented to it compared with a 20 per cent uptake by brain tissue. The combined activity of both tissues might effectively minimize any rise in ammonia concentration as a result of transfusion therapy. As these investigators point out, however, venous ammonia levels may not reflect the magnitude of ammonia presented to the brain through its arterial supply. Since a portion of the transfused blood is presented directly to the brain, the resultant local and temporary elevation could result in additional uptake by brain tissue. This would tend to perpetuate or accentuate the pre-existing metabolic disorder.

It appears that the use of bank blood in an early stage in its storage life is desirable in order to avoid aggravating an already present complication in the cirrhotic patient. Although the direct relationship between blood ammonia and hepatic coma is not categorically established, ammonia itself has been repeatedly shown to cause neurological disorders.[3, 6, 10] The evidence would seem to indicate that there is at least a correlation between ammonia and whatever other toxic factors are responsible for coma and other motor derangements in hepatic insufficiency.

SUMMARY AND CONCLUSIONS

1.. The ammonia content of bank blood stored at 4° C. has been shown to increase with time.

2. Preliminary data have been presented suggesting that the addition of sodium glutamate may have a depressing effect on the rate of ammonia formation in stored blood.

3. Contraindications to the use of old bank blood in the transfusion therapy of the cirrhotic patient have been discussed.

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OBSERVATIONS ON ADRENAL CORTICAL FUNCTION IN HYPERTENSIVE PATIENTS TREATED WITH POTASSIUM THIOCYANATE

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INTRODUCTION

THIOCYANATES have been used in the management of essential hypertension for many years.[1, 2] With the advent of the newer hypotensive agents, this drug has been relegated largely to the alleviation of severe headaches in hypertensive patients. In this, it has usually been successful.[3, 4]

The mode of action of thiocyanate remains unknown. It has been described as a sedative, as a direct vasodilator, and as a protoplasmic poison.[5] Further, it has been said to depress tissue metabolism by suppressing thyroid activity.[6] However, it has been shown that when the antithyroid effect has been countered by simultaneous administration of potassium iodide, the hypotensive action is unaltered.[7]

Thiocyanates have also been said to depress adrenal cortical function, and thus lower blood pressure. In 1931, Healy[8] described three patients treated with thiocyanates who experienced shock and coma. One of these developed pigment in the cubital fossae and mouth. He suggested that the gastrointestinal distress, rash, asthenia, and mental aberrations associated with thiocyanate therapy were similar to "hypoadrenia." Healy also noted that thiocyanate was found in the adrenal cortex at necropsy. Davis and associates[9, 10] have since demonstrated that thiocyanate therapy reduced the lipid content of the adrenal cortex, especially in the zona glomerulosa. Gubner[11] described a normal response to the Thorn test and a positive Robinson-Power-Kepler test in patients with essential hypertension treated with thiocyanates. He concluded that thiocyanates act on a renal mechanism rather than through the adrenal cortex.

In the present study, a group of five tests was used to assess the effect of thiocyanate (SCN) therapy on adrenal cortical function in patients with essential hypertension. It was considered that any changes in adrenal activity should be reflected in one or all of these tests.

METHODS

Three women patients with essential hypertension, aged twenty-eight, forty-two, and forty-five, acted as subjects. Each had been under observation for 5 to 7 years during

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which time systolic blood pressure readings ranged from 180 to 250 mm. Hg and diastolic readings from 110 to 130 mm. Hg. There was little evidence of cardiac or renal damage and all secondary causes of hypertension were excluded.

The subjects attended the Hypertension Clinic where weekly blood SCN levels and blood pressure readings were recorded by the same observers. The subject rested on a couch in a private cubicle while seven blood pressure readings were obtained over a forty-minute period. The double lines on Fig. 1 represent the highest and lowest systolic and diastolic pressures recorded at each visit. During the entire experiment each subject also received five grains of potassium iodide daily to prevent any hypothyroidism due to SCN administration.[8] Their usual diet was unaltered except for an additional 60 Gm. of carbohydrate on each of the three days preceding insulin-glucose tests.

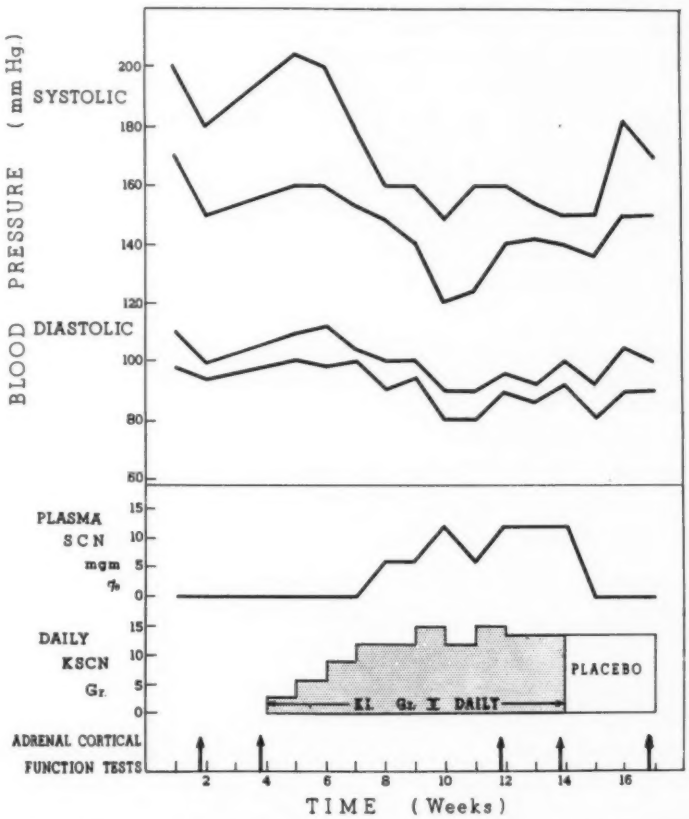

Fig. 1.—The effect of KSCN on blood pressure of Subject P. (Details in text.)

Two other patients who had been on prolonged SCN therapy for hypertension for 13 years and 5 years, respectively, were also investigated as to their adrenal cortical functions on a single occasion. In these patients a lowering of blood pressure had been achieved and maintained with SCN.

Adrenal cortical function was measured in the first three subjects by means of five tests. The serum sodium, twenty-four hour urinary output of sodium and 11-oxysteroids, the response to the insulin-glucose tolerance test, and the Oleesky water test were measured. The tests were carried out twice during the control period, and twice again after sufficient KSCN had been administered orally to maintain a blood SCN level of 6 to 12 mg. per cent for some four weeks. Following this, the subjects received a placebo in a similar dosage. After SCN had not been detected in the blood for a number of weeks, the tests were repeated.

The urinary 11-oxysteroids were estimated by the method of Gornall and MacDonald[12]; the insulin-glucose tolerance test was performed as described by Engel and Scott,[13] and the water test was carried out as described by Oleesky.[14] Serum and urinary sodiums were estimated by internal standard flame photometry. In the case of the two subjects who had been on prolonged SCN therapy, the insulin-glucose tolerance test was not done.

RESULTS

The administration of KSCN was associated with a significant lowering of blood pressure and alleviation of headache. Fig. 1 illustrates this effect in Subject P. These effects were reversed when placebo was substituted in Subjects K and P.

Two subjects, K and P, completed the experiment. The remaining subject (S) was relieved of headache with only a slight reduction in blood pressure. She died suddenly during the first series of tests administered while on treatment. A necropsy was performed and revealed death to be due to a ruptured "congenital" aneurysm of the right middle cerebral artery. There was generalized moderate arteriolosclerosis and minimal cardiac and renal hypertensive changes. Total adrenal weight was 10 Gm. Microscopic examination of the adrenal cortex stained with hematoxylin and eosin was considered to be normal.

Table I shows the data obtained with the five adrenal cortical function tests in the control, treatment, and placebo experimental periods for each subject.

TABLE I. 11-OXYSTEROID EXCRETION, SODIUM EXCRETION, OLEESKY WATER TEST, AND INSULIN-GLUCOSE TEST IN PATIENTS WITH ESSENTIAL HYPERTENSION, TREATED WITH POTASSIUM THIOCYANATE*

SUBJECT	URINE 11-OS MG./24 HR.			SERUM SODIUM MEQ./LITER			URINE SODIUM OUTPUT—MEQ./24 HR.		
	CONTROL	TREATMENT	PLACEBO	CONTROL	TREATMENT	PLACEBO	CONTROL	TREATMENT	PLACEBO
S	1.2	1.9		139	127		120	132	
	1.3			135			127		
K	1.9	2.8	1.6	137	141	140	103	132	160
	2.2	1.6		140	138		186	148	
P	1.5	1.0	1.5	139	139	140	107	153	117
	1.6	2.3		136	136		136	89	

	PEAK OLEESKY RESPONSE ML./MIN.			INSULIN-GLUCOSE TOLERANCE TEST (% OF FASTING BLOOD SUGAR)					
				NADIR			PEAK		
S	13.3	12.5		31			151		
	20.9			32			171		
K	7.7	11.2	13.8	57	53	38	105	111	124
	8.3	8.6		43	52		107	106	
P	8.3	14.8	10.3	28	20	27	161	116	103
	11.5	13.6		26	27		88	113	

*Where two values are given, these are separate determinations 7 days apart.

Serum sodium was found to be normal in all subjects, and unchanged by treatment with SCN. Subject S had a low level of serum sodium the day before death. The significance of this is uncertain as her urinary output of sodium was unchanged. Urinary excretion of sodium was not appreciably changed by therapy.

Values obtained in the Oleesky water test were moderately variable, but all were within the normal range and unaltered by SCN.

Insulin-glucose tolerance tests resulted in normal values for the insulin phase (the nadir) regardless of therapy. The glucose phase (the peak) in Subjects K and P was at the lower limit of normal set by Engel and Scott[13] but was unchanged by the treatment.

The 11-oxysteroid excretion during control periods was quite constant. The excretion for Subject P during administration of placebo was similar to her control excretion, but in Subject K, excretion while on placebo was slightly lower than her control levels. While on SCN, K and P each had a high and low value as compared to the control values. The one estimation done on Subject S while on SCN was elevated above her control level. All the figures, however, are within the normal range given by Gornall and MacDonald,[12] except for one high value of 2.8 mg. per 24 hours in Subject K while on treatment. The value seven days later, while still on treatment, was normal.

In Table II are shown the data from the two subjects who had been on prolonged SCN therapy. As will be seen, all values were within normal limits, indicating that prolonged SCN therapy had had no effect on adrenal cortical function.

TABLE II. 11-OXYSTEROID EXCRETION, SODIUM EXCRETION, SERUM SODIUM, AND OLEESKY TEST IN TWO PATIENTS ON PROLONGED KSCN THERAPY

SUBJECT	SERUM Na MEQ./L.	URINE Na MEQ./24 HR.	11-OXYSTEROIDS MG./24 HR.	PEAK OLEESKY RESPONSE ML./MIN.
C	141	127	1.0	7.7
Kg	142	102	1.15	7.0

DISCUSSION

In acute experiments, Pines and Perera[14] demonstrated a small sodium diuresis in hypertensive subjects treated with SCN. Gubner[11, 15] reported that SCN therapy produced an increased salt excretion with no change in serum sodium levels. The data presented here agree in that serum sodium levels are unaltered. The finding of normal serum sodium values with unaltered sodium excretions suggests normal adrenal function.

The insulin-glucose tolerance test is a modification of the insulin test. Only the latter has been used in hypertensive subjects. De Takats[16] found 24 out of 50 hypertensive patients to be resistant to intravenous insulin, while Olsen[18] observed that a group of 38 patients with essential hypertension gave a normal response. Mirsky[19] demonstrated the response to hypoglycemia in patients with essential hypertension to be significantly lower than normal. Subject S exhibited a normal response to hypoglycemia, while Subjects K and P showed a slight defect in the response to hypoglycemia, which, however, was unaltered by SCN.

The formaldehydogenic corticosteroid excretion has been investigated in hypertensive patients by several authors. Daughaday,[20] Corcoran,[21] and

Tobian[22] found the excretion to be normal, with the occasional patient excreting somewhat larger amounts. The control and placebo observations presented here agree with these findings.

The 11-oxysteroid excretion in hypertensives treated with SCN has been estimated in four patients by Davis and associates.[23] These authors found that after two weeks of therapy, two patients had elevated levels, one had decreased levels, and the other was unchanged. After four months of therapy the steroid excretion was elevated in two, but the others were at control levels. The present data reveal three increased and two decreased excretion values in five observations on three subjects. On the whole, our results indicate that there is no consistent change in urinary corticosteroid excretion when SCN is administered to hypertensive patients.

The state of hypoadrenalism is associated with hypotension, diminished insulin response to glucose, decreased urinary excretion of corticosteroids, sodium diuresis and hyponatremia, and diminished response to a water load as well as many other features. In this study, the administration of SCN produced a lowering of blood pressure which was not correlated with any consistent changes in these parameters of adrenal cortical activity.

SUMMARY AND CONCLUSIONS

Administration of SCN did not alter indices of adrenal cortical activity, although the usual blood pressure reductions were nevertheless obtained. It may therefore be concluded that the hypotensive action of SCN is not due to alteration of adrenal cortical activity.

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THE PRACTICAL APPLICATION OF THE RAPID INTRAVENOUS GLUCOSE TOLERANCE TEST IN VARIOUS DISEASE STATES AFFECTING GLUCOSE METABOLISM

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THE most widely accepted procedure employed in the clinical study of unexplained glycosuria is the oral glucose tolerance test, a test which depends upon the intestinal absorption of glucose. The amount and rate of absorption of glucose by the alimentary tract introduces an uncontrollable variable in the oral test. Previous studies have shown that one hour after the giving of 100 Gm. of glucose orally to normal and diabetic subjects, 22 to 68 Gm. of the administered glucose may be recovered by washing the stomach without meaningful variation of the glucose tolerance test.[1] Furthermore, the nausea experienced by some individuals after the ingestion of glucose alters the oral glucose tolerance test.[2] In normal subjects, repetition of the oral test resulted in curves which, in the same individuals, differed in the maximum glucose concentration attained as well as in the time of occurrence and duration of the maximum value.[3, 4] This is apparently related to variations in the amount and rate of transfer of glucose through the alimentary tract.

To avoid the variable factor of absorption of glucose from the alimentary tract, the rapid intravenous glucose tolerance test was employed. This test has been widely studied.[3, 5, 6] However, varying quantities of glucose and unsuitable controls have sometimes been used, causing difficulty in establishing a standard test. It is our purpose to present a simple test for routine use.

MATERIAL AND METHOD

All subjects tested had been on an adequate diet containing at least 150 Gm. of carbohydrate daily for at least one week. For adults, 25 Gm. of glucose as a 30 per cent solution in distilled water was given intravenously within four minutes. It is suggested that children and adults under 125 pounds be given 0.5 Gm. per kilogram of body weight of glucose. Blood specimens were drawn before and 68 minutes *after* the administration of the glucose. Determination of the capillary blood glucose was done by the Horvath and Knehr Modification[7] of the Folin-Malmros micromethod.[8] Blood glucose values are expressed as milligrams per cent at 68 minutes in excess of the fasting blood glucose value.

The present study includes 386 individuals, of whom 103 are normal controls. This control group consisted of normal healthy males ranging in age from 25 to 50 years with

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no family or personal history of diabetes mellitus. The test was repeated on 20 of the normal subjects within one month. Fourteen patients had untreated hyperthyroidism and, of the remaining patients, 26 had mild dietary controlled diabetes mellitus—that is, when uncontrolled by diet, they had glycosuria and a decreased oral tolerance test—and 13 had severe diabetes mellitus. Twenty-six patients had moderately severe cirrhosis of the liver, 28 had active infectious hepatitis, and 68 had inflammatory disease states including uremia due to chronic glomerulonephritis[19] or hypertensive renal disease[10] and infectious diseases.[39] In addition, the test was performed on 9 patients with obesity and 8 suffering from malnutrition.

An oral glucose tolerance test was done on 144 individuals. A single dose of 100 Gm. of glucose in water was given to each subject maintained on an adequate diet containing at least 150 Gm. of carbohydrate per day for at least one week. The control group included 115 normal healthy males of ages 23 to 55 years who had no family or personal history of diabetes mellitus. Fourteen patients had untreated hyperthyroidism and 15 subjects had mild controlled diabetes mellitus (no insulin required). The venous blood glucose was determined by the method of Benedict.[9]

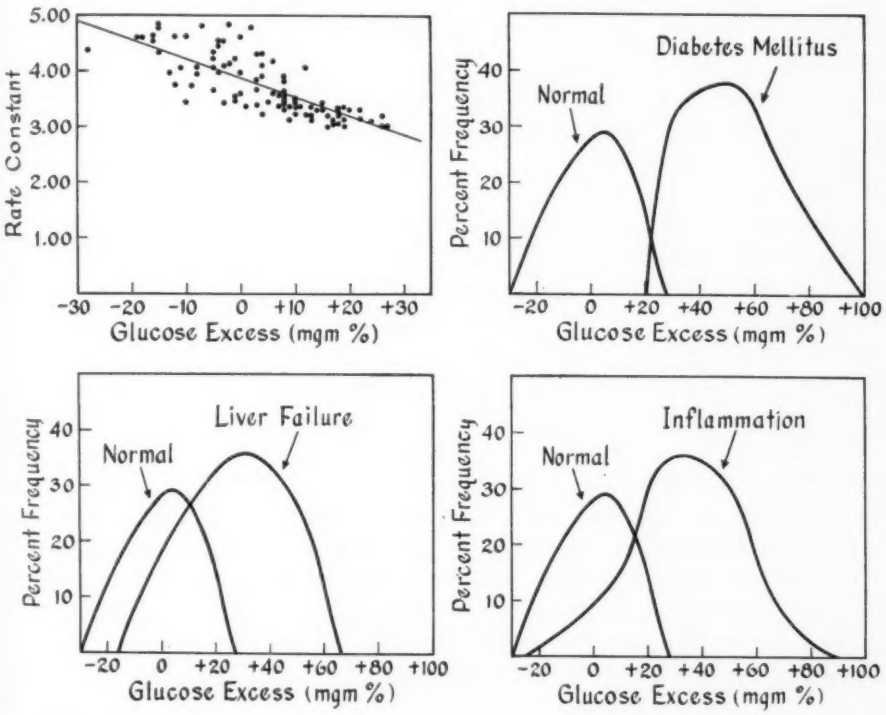

Fig. 1.—A correlation of rate constant with the 68-minute glucose excess and frequency distribution curves of normals as compared with patients having diabetes mellitus, liver failure, and inflammation.

RESULTS

The relationship between the rate constant of glucose and the 68-minute glucose excess value is shown in Fig. 1 with a significant correlation coefficient (r) of 0.81. The rate constant of glucose was determined as in previous studies,[10-12] and analyses of the differences between the rate constants in normal and various disease states revealed these differences to be significant (Table I).

TABLE I. THE BLOOD GLUCOSE EXCESS (MG. PER CENT) AT 68 MINUTES AND THE RATE CONSTANT DURING THE RAPID INTRAVENOUS GLUCOSE TOLERANCE TEST

SUBJECT	NO.	GLUCOSE EXCESS (MG. PER CENT) AT 68 MINUTES			RATE CONSTANT (PER CENT/MINUTE)		
		RANGE (MG. PER CENT)	MEAN AND S. D.	P.	RANGE	MEAN AND S. D.	P.
Normal	103	−28 to +26	+4.6 ± 12.1		3.00 to 4.84	3.72 ± 0.54	
a. First test	20	−12 to +24	+4.6 ± 11.0		3.20 to 4.84	3.70 ± 0.55	
b. Repeat test	20	−19 to +22	+5.1 ± 11.5	>0.2	3.20 to 4.74	3.76 ± 0.50	>0.2
Hyperthyroidism	15	−21 to +23	+0.3 ± 14.3	>0.2	3.04 to 11.17	5.05 ± 2.22	<0.001
Diabetes mellitus	39	+20 to +102	+50.4 ± 18.0	<0.001	0.23 to 2.46	1.56 ± 0.60	<0.001
Cirrhosis	38	−17 to +66	+30.0 ± 19.0	<0.001	0.70 to 4.07	2.20 ± 0.78	<0.001
Viral hepatitis	28	−7 to +59	+27.9 ± 14.9	<0.001	1.21 to 4.33	2.60 ± 0.45	<0.001
Inflammation	68	−34 to +97	+37.4 ± 22.6	<0.001	0.42 to 4.77	2.02 ± 0.86	<0.001
Obesity	9	+13 to +41	+29.8 ± 10.6	<0.001	1.37 to 2.56	2.05 ± 0.39	<0.001
Starvation	8	+44 to +89	+58.9 ± 14.9	<0.001	0.86 to 2.58	1.77 ± 0.49	<0.001

The blood glucose excess at 68 minutes and the rate constant of normal subjects and those having diseases affecting glucose metabolism are shown in Table I. The glucose excess of the normal group ranged from −28 to +26 mg. per cent with a mean of 4.6 ± 12.1 mg. per cent. The intravenous test was repeated on 20 normal subjects, and a comparison of the glucose excess at 68 minutes of the initial and repeat test were similar with means of 4.6 ± 11 and 5.1 ± 11.5 mg. per cent, respectively. These means do not differ significantly (Table I).

A comparison of the mean of the glucose excess of the normal group with the mean excess obtained in each group representing diabetes mellitus, hyperthyroidism, inflammation, moderately severe liver disease, obesity, or malnutrition revealed significant differences except for hyperthyroidism (Table I). The nonglucose-reducing substances were determined on the fasting and 68-minute blood glucose specimens by fermentation in patients with uremia. The nonglucose reducing substances were found to be relatively constant in the fasting and 68-minute values. Graphically illustrating the separation of two groups, Fig. 1 shows a frequency distribution of the 68-minute glucose excess value of the normal controls and diabetes mellitus patients. Only one patient with diabetes mellitus had a glucose excess value less than 26 mg. per cent, while a comparison between the frequency distribution curves of the normal controls and of liver disease and inflammation (Fig. 1) showed a partial superimposition of the normal curve upon these various disease states.

The oral glucose tolerance test in 115 normal subjects is shown in Table II. The maximum value attained during the test was similar at 30 and at 60 minutes. The most significant value was at 120 minutes where all blood glucose values were below 110 mg. per cent. During the oral test a maximum of only 120 mg. per cent was found in 40 subjects, or 34 per cent. A comparison of the rapid intravenous and oral glucose tolerance tests in hyperthyroid and mild controlled diabetic states is shown in Tables III and IV. With the rapid intravenous test in hyperthyroidism, all patients had 68-minute glucose excess values in the normal range with a mean of 0.3 ± 14.3 mg. per cent. However, with the oral test in hyperthyroidism, only 9 of 14 patients had 120-minute

glucose values below the normal maximum of 110 mg. per cent. In mild controlled diabetes mellitus, the rapid intravenous test was abnormal in all patients with a mean 68-minute glucose excess of 42 ± 11 mg. per cent, while the oral glucose test showed 4 of 15 patients with values below 110 mg. per cent at 120 minutes.

TABLE II. THE 100 GM. STANDARD ORAL GLUCOSE TOLERANCE TEST IN NORMAL SUBJECTS

TIME (MINUTES)	BLOOD GLUCOSE RANGE (MG. PER CENT)	NO.	MEAN (MG. PER CENT)	PER CENT
0		115	84 ± 10	100.0
30	<100	12	84 ± 16	10.4
	100-149	15	125 ± 15	47.8
	150-199	41	169 ± 13	35.7
	>200	7	232 ± 42	6.1
60	<100	24	78 ± 14	20.7
	100-149	55	123 ± 16	48.0
	150-199	29	175 ± 10	25.2
	>200	7	216 ± 14	6.1
120	<74	22	61 ± 13	19.1
	75- 99	76	89 ± 7	66.1
	100-110	17	104 ± 4	14.8
180	<74	84	59 ± 5	73.0
	75- 99	26	84 ± 6	22.6
	100-110	5	103 ± 2	4.4

TABLE III. A COMPARISON OF THE RAPID INTRAVENOUS AND ORAL GLUCOSE TOLERANCE TEST IN MILD DIABETES MELLITUS

	RAPID INTRAVENOUS TEST (MG. PER CENT)			ORAL GLUCOSE TOLERANCE (MG. PER CENT)				
SUBJECT	0 (MINUTES)	68-MINUTE EXCESS	RATE CONSTANT (PER CENT/MIN.)	0	30	60 (MINUTES)	120	180
W. D.	105	38	2.03	82	96	132	140	73
O. T.	98	45	2.27	88	163	193	102	72
D. H.	105	38	1.83	92	130	168	155	108
M. B.	94	42	2.22	82	135	164	103	90
B. O.	80	43	2.14	75	165	208	123	50
W. B.	94	43	1.79	77	175	128	107	54
A. P.	102	28	1.87	80	139	178	150	90
W. S.	185	29	2.07	94	182	318	316	236
E. C.	145	35	2.32	98	173	174	154	91
J. L.	113	45	1.77	108	306	308	113	90
F. S.	89	55	1.23	75	108	150	130	76
A. W.	94	42	1.64	82	196	140	114	73
B. M.	115	28	2.10	108	168	133	122	58
E. T.	95	48	1.79	96	152	150	117	63
J. E.	111	72	1.09	99	261	285	110	109
Mean	108 ± 27	42 ± 11	1.88 ± 0.33	89 ± 11	170 ± 52	188 ± 63	134 ± 55	89 ± 43

DISCUSSION

The rapid intravenous glucose tolerance test has been recommended in the past.[3, 5, 6, 13-15] However, lack of suitable controls and small numbers of subjects characterized previous studies. With a constant exponential fall of blood glucose excess during the intravenous test, it had previously been observed that in normal subjects the 60-minute value was 20 mg. per cent or less in excess of the fasting glucose value.[3, 5, 6, 13] The 68-minute blood glucose value was selected in the present study because maximum separation between

TABLE IV. A COMPARISON OF THE RAPID INTRAVENOUS AND ORAL GLUCOSE TOLERANCE TEST IN HYPERTHYROIDISM

	RAPID INTRAVENOUS TEST (MG. PER CENT)			ORAL GLUCOSE TOLERANCE (MG. PER CENT)				
SUBJECT	0 (MINUTES)	68-MINUTE EXCESS	RATE CONSTANT (PER CENT/MIN.)	0	30	60 (MINUTES)	120	180
W. B.	113	–21	5.08	88	207	140	70	77
P. D.	113	–10	3.85	89	149	114	114	74
P. M.	108	–10	8.59	75	150	135	115	57
M. K.	115	–16	4.33	86	140	135	94	87
J. B.	100	+23	3.04	76	145	159	137	78
M. P.	90	–4	4.47	79	155	154	97	75
R. R.	88	–2	4.20	78	180	193	102	71
W. L.	87	+18	3.08	76	172	193	152	100
E. R.	83	+18	3.30	60	78	189	106	71
G. G.	88	+23	3.08	75	158	83	55	68
H. K.	92	–5	4.89	73	202	224	122	43
J. F.	101	–3	4.41	77	212	224	76	62
R. M.	105	–5	11.17	74	137	108	87	72
L. S.	98	+7	4.62	91	177	96	78	82
Mean	99 ± 7	0.3 ± 14	5.05 ± 2.22	78 ± 11	161 ± 35	153 ± 45	100 ± 27	73 ± 11

our normal and abnormal groups occurred at this time. The rapid intravenous glucose tolerance test was repeated in 20 patients within one month and, on comparison of the glucose excesses at 68 minutes, the values were similar and the differences were statistically insignificant. The acceptance of any procedure is dependent upon its reproducibility under uniform conditions; however, repeated oral glucose tolerance tests lack any parallelism.[3, 4] This variability in repeated oral tests in the same subject is the result of an irregular rate of alimentary glucose absorption and tends to invalidate the procedure. The loss of glucose in the urine during the intravenous test was negligible in normal subjects as only 0.2 to 1.5 Gm. was excreted. No significant change in blood volume following intravenous injection of 25 Gm. of glucose as a 50 per cent solution was found by Pijoan and Gibson[16] using Evans Blue dye.

The above application of the rapid intravenous glucose tolerance test gave a sharp separation of the dietary-controlled patients with mild diabetes mellitus from the normal individuals, and this is best shown by the frequency distribution curves of Fig. 1. In contrast, the oral glucose tolerance test was normal in 4 of 15 patients with known controlled mild diabetes mellitus (Table III), which is in agreement with previous studies.[15] Because of the variability of glucose absorption from the alimentary tract, the oral glucose tolerance test is limited as a diagnostic aid in mild diabetes mellitus. In moderate to severe diabetes mellitus the oral test adds no information to that obtained by determining the fasting blood glucose. Because of the above, and the variability of the alimentary absorption of glucose, Soskin[15] has labeled the oral glucose test a "practically worthless" procedure and recommends the rapid intravenous test.

It is generally accepted that hyperthyroidism accelerates the removal of glucose from the blood.[17, 18] In the present study all patients with hyperthyroidism who were studied with the rapid intravenous glucose tolerance test had normal glucose excess values at 68 minutes. In contrast, when the oral

test was used, only 9 of 14 patients with hyperthyroidism had normal tolerance tests (Table IV). An impairment in carbohydrate utilization should not be anticipated as implied by the oral test in hyperthyroidism. In the hyperthyroid state the direct action of thyroxine on tissue is to increase oxygen uptake[19] as well as to increase the rate of removal of glucose.[17, 18] Other studies in hyperthyroidism have revealed that the oxidation or utilization of glucose is either normal or increased.[20, 21]

A diminished glucose tolerance is known to be present in inflammatory states,[3, 6] and is probably related to weight loss, an inadequate nutritional intake, or a relative insulin insensitivity.[22, 23] The adverse effects of inflammation upon glucose utilization were demonstrated by an abnormal intravenous tolerance test. The rapid intravenous glucose tolerance test was abnormal in a majority of patients with active liver disease. This is in agreement with previous work.[13, 24] In renal insufficiency with uremia, a diminished rapid intravenous glucose tolerance was found and it may be related either to weight loss, inflammation, an inadequate food intake, or the effects of disease. In these patients the nonglucose reducing substances remained relatively constant during the test. In general the clinical interpretation of an abnormal glucose tolerance requires an awareness that inflammation, uremia, active liver disease, malnutrition, and obesity may be responsible for such abnormality.

SUMMARY

A simple and rapid test was demonstrated with use of the 25 Gm. rapid intravenous glucose tolerance test in the study of unexplained glycosuria. The variable factor of absorption of glucose from the alimentary tract was thus avoided.

In a control group of 103 normal men, the glucose excess at 68 minutes during the rapid intravenous glucose tolerance test ranged from –28 to +26 mg. per cent of glucose. In 20 normal men the glucose excesses at 68 minutes were almost identical on repetition of the rapid intravenous glucose tolerance test in the same subjects. Thirty-nine patients with known diabetes mellitus showed a sharp separation from the normal control group with a mean excess of 50.4 mg. per cent and range of 20 to 102 mg. per cent at 68 minutes. In untreated hyperthyroidism the glucose excess at 68 minutes during the rapid intravenous glucose tolerance test was 20 mg. per cent or less in all patients.

In the infectious or inflammatory states studied (severe liver disease, infectious disease states, inflammation, and uremia), the glucose excess at 68 minutes during the test was above 20 mg. per cent in the majority of cases.

Values of glucose excess greater than 26 mg. per cent at 68 minutes are considered abnormal by the authors and indicate a diminished glucose tolerance.

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THE INTRINSIC VISCOSITY OF SYNOVIAL FLUID HYALURONIC ACID

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THE low relative viscosity of joint fluid in rheumatoid arthritis has stimulated study of synovial fluid hyaluronic acid. The viscosity of synovial fluid is determined by the concentration and the degree of polymerization of the hyaluronic acid. Synovial fluid viscosity and the concentration of hyaluronic acid have been found to be lowered in a variety of disease states.[1] The qualitative appearance of the mucin clot which forms on acidification of synovial fluid has been considered to be indicative of the degree of polymerization of the hyaluronic acid.[2] Since the mucin clot is a combination of hyaluronic acid and a variable amount of protein, it is difficult to interpret the abnormalities in the mucin clot which have been observed in a variety of joint diseases by Ropes and Bauer.[2] A quotient obtained by dividing the square root of the relative viscosity of the synovial fluid by the hyaluronic acid concentration was considered by Ragan and Meyer[3] to be indicative of the degree of polymerization, and this quotient was found to be low in patients with rheumatoid arthritis compared to normal joint fluid obtained at postmortem. The extent of the decrease in polymerization was thought to correlate with the severity of the arthritis. On the basis of this data it was postulated that patients with rheumatoid arthritis form an increased amount of poorly polymerized hyaluronic acid, and that this is a primary fault in the disease.[3]

In view of this observation, a study was undertaken to measure the degree of polymerization of synovial fluid hyaluronic acid, as reflected by changes in the intrinsic viscosity† of the hyaluronic acid. The aim of the study has been to determine if poor polymerization of synovial hyaluronic acid is a specific change in rheumatoid arthritis, and to see if any of the clinical characteristics of that disease correlate with observable alterations in the hyaluronic acid. Since this study was undertaken, reports have been published

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†Relative viscosity is the viscosity of a solution as related to a standard of reference, usually water (= 1). Specific viscosity (relative viscosity —1) is the amount of increase in viscosity. Intrinsic viscosity is the increase in viscosity divided by the concentration, extrapolated to zero concentration. The intrinsic viscosity measures in absolute units the space (volume) required by a molecule during flow in the limit of extreme dilution, and hence for a given type of polymer is related directly to the degree of polymerization.

of the finding that the decrease in polymerization of synovial hyaluronic acid was not specific for rheumatoid arthritis.[4, 5] Our data confirm these findings with use of different methods and add observations on the apparent lack of correlation of the degree of change in the hyaluronic acid with the clinical aspects of the patients with rheumatoid arthritis. In addition, observations were made of the influence of steroid therapy on the intrinsic viscosity of synovial fluid hyaluronic acid.

METHODS

All patients were seen by the principal investigator and were studied only when a definite diagnosis could be established. Synovial fluid was obtained by aspiration from knee joints, usually without anesthesia, or following ethyl chloride spray. When sufficient fluid was available, the relative viscosity was determined before further handling. In a few instances, when insufficient quantities of fluid were found by the usual technique, a "joint wash" was obtained by injecting about 20 ml. of physiologic saline into the joint and then withdrawing it. In order to determine if this procedure altered the hyaluronic acid, studies were done on "wash" obtained from knees after maximum evacuation of an effusion. The intrinsic viscosity of the hyaluronic acid found in the "wash" did not differ from that of the hyaluronic acid removed previously from the same joints in the free fluid (Fig. 1).

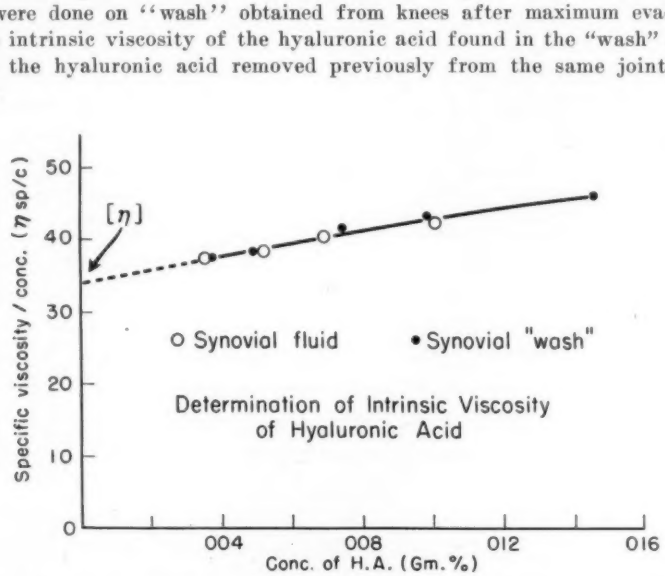

Fig. 1.—Plot used for determination of intrinsic viscosity of hyaluronic acid. Example shown includes points found for synovial fluid and "wash" (see text) from same patient.

Samples were kept frozen until ready for use, and were then dialyzed for 24 hours in the cold against a buffer consisting of 0.05 M PO_4 at pH 7.0 with 0.0125 M NaCl. Viscosity and concentration determinations were done on separate aliquots of the dialyzed fluid. The hyaluronic acid concentration was measured after removal of most of the protein. To accomplish this, 0.5 ml. of fluid was diluted to 2.0 ml. with water and 5.0 ml. of chloroform was added. The mixture was agitated on a mechanical shaker for one hour, centrifuged, and the supernatant fluid separated from the protein coagulum at the interface. It was found that this procedure removed approximately 75 per cent of the protein in the synovial fluid, but that solutions of hyaluronic acid were not affected, and that hyaluronic acid added to synovial fluid or serum was quantitatively recovered in the supernatant. The hyaluronic acid concentration in the supernatant was determined by duplicate measurement of the uronic acid content by the carbazole method of Dische.[6] In this determination 10 and 20 mcg. of glucuronolactone were added to separate aliquots

of each fluid in order to establish the recovery of added standards. The precision of this method was found to be ± 5 per cent for single determinations. The hyaluronic acid concentration was calculated with use of an estimate of 43 per cent for its uronic acid content and expressed as grams per 100 milliliters in further calculations.

The intrinsic viscosity of the hyaluronic acid of each dialyzed fluid was determined by serial measurements of the specific viscosity (relative viscosity—1) at several concentrations. Dilutions were made with the dialysis buffer. All viscosity determinations were done in a water bath at 37° C., with an Ostwald viscometer of 2 ml. capacity, calibrated with buffer. Each fluid was run at several relative viscosities between 1.20 and 1.40. At this concentration range a straight line relationship was found for specific viscosity divided by concentration ($\eta sp/C$) plotted against concentration. The intrinsic viscosity was determined by extrapolating this plot to zero concentration (Fig. 1). Since all fluids were run in this manner, the salt concentration, pH, flow times, pressure, and the range of concentrations of hyaluronic acid studied were the same for each specimen.

RESULTS

Intrinsic viscosity of hyaluronic acid (H.A.) of synovial fluid.—Nine synovial fluids obtained at postmortem from undiseased knees showed intrinsic viscosities of the H.A. ranging from 54 to 91 units, averaging 69.3 with standard error of the mean of 4.2 units (Table I). Synovial fluid from 25 patients with rheumatoid arthritis, untreated or treated with aspirin and/or physical measures, showed intrinsic viscosity ranging between 5 and 69 units, averaging 37.6 units. Only two of these fluids showed an intrinsic viscosity over 55, twenty were below 50 (Table II). Fluid from 6 patients with traumatic arthritis showed intrinsic viscosities between 27 and 38, averaging 32.5 units

TABLE I. RELATIVE VISCOSITY, HYALURONIC ACID CONCENTRATION, AND INTRINSIC VISCOSITY IN NORMAL SYNOVIAL FLUID OBTAINED AT POSTMORTEM

RELATIVE VISCOSITY	CONCENTRATION HYALURONIC ACID (GM. %)	INTRINSIC VISCOSITY
	0.422	91
40.0	0.194	85
	0.189	71
121.0	0.099	70
58.0	0.168	68
	0.146	63
55.3	0.126	62
3.9	0.035	60
	(0.013)	54
		(Mean 69.3 ± S.E.M. 4.2)

The concentrations given in parentheses were obtained from joint "wash."

(Table III). Four fluids from cases of acute pyogenic arthritis revealed intrinsic viscosities between 54 and 70 units; the only case of tuberculous arthritis studied had an intrinsic viscosity of 4 units. Five knees affected by acute systemic lupus erythematosus were studied; intrinsic viscosities ranged between 21 and 83 units, averaging 54.2, with two fluids falling below 40. Four osteoarthritic knees showed intrinsic viscosities between 41 and 66, averaging 49.8 units. Three patients with acute exacerbations of gouty arthritis of the knee showed intrinsic viscosities of the hyaluronic acid ranging between 37 and 72, averaging 50.7. A series of fluids from patients with other forms of arthritis were studied, all having intrinsic viscosities below 40 units;

TABLE II. RELATIVE VISCOSITY, HYALURONIC ACID CONCENTRATION, AND INTRINSIC VISCOSITY IN SYNOVIAL FLUID FROM 25 PATIENTS WITH RHEUMATOID ARTHRITIS

RELATIVE VISCOSITY	CONCENTRATION HYALURONIC ACID (GM. %)	INTRINSIC VISCOSITY
5.3	0.036	69
8.4	0.042	59
8.4	0.036	55
15.4	0.099	55
24.6	0.102	54
9.9	0.060	48
7.1	0.067	47
13.2	0.117	46
7.1	0.056	38
4.9	0.055	37
15.6	0.127	36
	0.035	36
2.6	0.036	35
7.9	0.079	35
5.9	0.104	34
	(0.018)	34
	0.111	33
	0.100	32
11.2	0.094	32
47.5	0.164	32
25.4	0.176	27
10.9	0.141	26
18.3	0.126	23
19.6	0.242	10
	(0.019)	5
		(Mean 37.6)

The concentrations given in parentheses were obtained from joint "wash."

these included three cases of rheumatic fever, two of scleroderma and both knees of a patient with Touraine-Solente-Golé syndrome (idiopathic hypertrophic osteoarthropathy with pachyderma). Synovial fluid from a patient with myxedema showed the highest concentration of hyluronic acid observed (0.359 Gm. per cent) and the highest relative viscosity of the native fluid (868), but the intrinsic viscosity was below that observed in normals (42 units) (Table III).

Effect of Altering the Salt Concentration.—It has been established that the viscosities of solutions of hyaluronic acid vary when the concentrations of ions in the solution are altered,[1] presumably due to variations in the hydration of particles of hyaluronic acid. In order to determine if the observed variations in intrinsic viscosity might be an artifact of the particular conditions chosen, a series of fluids with widely varying intrinsic viscosities from patients with different types of arthritis were redialyzed to alter the salt concentrations. The observed intrinsic viscosities rose after dialysis against buffer of lower salt concentration and fell when the salt concentration was raised. Changes of similar magnitude were observed in all fluids studied.

Relationship of Intrinsic Viscosity of the H.A. to Features of the Disease in Patients With Rheumatoid Arthritis.—No correlation was found between the intrinsic viscosity of the synovial fluid and the duration of the disease in patients with rheumatoid arthritis. The severity of the inflammation of the knee was estimated clinically by the degree of tenderness, swelling, and warmth of

TABLE III. RELATIVE VISCOSITY, HYALURONIC ACID CONCENTRATION, AND INTRINSIC VISCOSITY IN SYNOVIAL FLUID IN VARIOUS DISEASES

DIAGNOSIS	RELATIVE VISCOSITY	CONCENTRATION HYALURONIC ACID (GM. %)	INTRINSIC VISCOSITY
Osteoarthritis	36.7	0.124	66
Osteoarthritis	450.0	0.222	49
Osteoarthritis	298.0	0.207	43
Osteoarthritis		(0.0004)	41
Gout	3.6	0.325	72
Gout		(0.0094)	43
Gout	73.5	0.180	37
Pyogenic arthritis	4.8	0.029	70
Pyogenic arthritis	59.3	0.143	61
Pyogenic arthritis	5.3	0.043	61
Pyogenic arthritis	26.0	0.138	54
Tuberculous arthritis	2.3	0.172	4
Traumatic arthritis		0.065	38
Traumatic arthritis	3.8	0.043	36
Traumatic arthritis	3.5	0.036	34
Traumatic arthritis		0.119	31
Traumatic arthritis	4.6	0.061	29
Traumatic arthritis		0.080	27
Systemic lupus eryth.		(0.0212)	83
Systemic lupus eryth.	59.8	0.101	66
Systemic lupus eryth.	307.0	0.163	62
Systemic lupus eryth.		(0.0081)	39
Systemic lupus eryth.	8.8	0.144	21
Scleroderma		(0.0021)	36
Scleroderma		(0.0077)	13
Rheumatic fever	12.1	0.122	33
Rheumatic fever	18.2	0.186	31
Rheumatic fever		0.0959	29
Touraine solente golé	39.6	0.112	39
	20.5	0.145	31
Myxedema	868.0	0.359	42

The concentrations in parentheses were obtained from joint "wash."

the joint and by the total white cell count and total number of polymorphonuclear leukocytes per cubic millimeter in the fluid, but none of these indices showed any correlation with the level of the intrinsic viscosity. Total protein determinations were done on a series of fluids from patients with a variety of diseases, and no relationship was found between the protein content of the fluid (which varied between 0.8 and 6 Gm. per cent) and the observed intrinsic viscosity of the H.A.

X-rays of the knee were obtained in 14 patients with rheumatoid arthritis and the degree of cartilage and bone destruction was estimated carefully for each joint, with grading on a 0 to 4 basis. These x-ray findings were then compared to the synovial fluid findings, but again no correlation was found with the intrinsic viscosity of the H.A.

Effect of Therapy With Anti-inflammatory Hormones on the Intrinsic Viscosity of the Hyaluronic Acid.—Eight patients with rheumatoid arthritis were studied before treatment with steroid and again at the time of maximal sup-

pression of the clinical and laboratory manifestations of the inflammatory process. A marked improvement in these findings occurred in each of these patients, in all joints in the five who received oral steroid, and locally in the three who received intra-articular injections. The intrinsic viscosity of the H.A. was low in all patients before treatment, averaging 35 units; when the inflammation was suppressed, the synovial fluid H.A. was found to have increased in intrinsic viscosity in 6 patients, the increase ranging from 1 to 15 units and averaging 5 units in these patients; one patient showed no change, and one a decrease in intrinsic viscosity of 17 units (Table IV). The relative viscosity of the undiluted synovial fluid was found to have increased following treatment in two cases in which sufficient fluid was obtained to make this determination. This increase was found to be more closely correlated with increases in the concentration rather than changes in the intrinsic viscosity of the hyaluronic acid.

TABLE IV. EFFECT OF STEROID THERAPY ON INTRINSIC VISCOSITY OF SYNOVIAL FLUID HYALURONIC ACID

STEROID ADMINISTERED	BEFORE THERAPY				AFTER THERAPY			CHANGE IN INTRINSIC VISCOSITY
	ROUTE	CONC. H. A.*	RELATIVE VISCOSITY	INTRINSIC VISCOSITY	CONC. H. A.*	RELATIVE VISCOSITY	INTRINSIC VISCOSITY	
Prednisolone	Oral	0.055	4.9	38	(0.0064)		44	+6
Prednisolone	Oral	0.100		32	(0.0068)		41	+9
Prednisone	Oral	0.164	47.5	32	(0.0203)		37	+5
Prednisone	Oral	0.111		33	(0.0339)		34	+1
Cortisone	Oral	(0.0183)		34	(0.0175)		34	0
Hydrocortisone	Intra-artic.	0.102	24.6	54	0.200	38.6	37	-17
Hydrocortisone	Intra-artic.	0.126	18.3	23	0.077	16.7	38	+15
Hydrocortisone	Intra-artic.	0.104	5.9	34	0.184	77.4	45	+4
		Average		35			39	

*Concentrations in parentheses were obtained from joint "wash."

DISCUSSION

Compared to normal human synovial fluid, there is a considerable decrease in the degree of polymerization of the hyaluronic acid, as reflected by measurement of its intrinsic viscosity, in a great proportion of patients with rheumatoid arthritis. A similar change has been found in a group of patients with other types of arthritis, including traumatic arthritis, osteoarthritis, acute gout, rheumatic fever, and others. These findings are in agreement with the reports of other investigators,[4, 5] except that the values for intrinsic viscosity of normal synovial fluid hyaluronic acid are considerably higher than previously reported for two normal human fluids.[4] The number of cases observed in this study is too small to discuss the distribution of the changes found in the various diseases, but it is evident that alteration of the intrinsic viscosity of synovial hyaluronic acid as revealed by these methods is not specific for any

single type of arthritis. In addition, no correlation could be demonstrated between the observed alteration in the intrinsic viscosity of the hyaluronic acid and clinical aspects of the rheumatoid arthritis in the patients studied. Comparisons were made with the duration of the disease and the degree of radiologically demonstrable bone and cartilage destruction. These findings are contrary to those of Ragan and Meyer[3] who noted a correlation between the degree of depolymerization and their clinical estimation of the "severity of the arthritis," and do not seem to support the suggestion that a decrease in the polymerization of hyaluronic acid is a prime fault in rheumatoid arthritis.[3]

It is of interest that the changes in intrinsic viscosity were not found to correlate with the severity of the clinical or laboratory evidence of inflammation of the joint, suggesting that this change is not a nonspecific result of the inflammatory process in the manner of the so-called "acute phase reactants" seen in the serum. Marked suppression of the inflammation with steroid hormones resulted in very little increase in the observed intrinsic viscosity of the hyaluronic acid in seven of eight cases, and in no case did it cause a return to near normal levels, although clinical findings and laboratory data, including synovial biopsy, showed marked reversal of the inflammatory changes. This finding supports the contention that the observed alteration in intrinsic viscosity of the hyaluronic acid is not secondary to the inflammatory process.

Other observers, using different methods, have reported an increase in the degree of polymerization of synovial fluid hyaluronic acid following suppression of inflammation by hormone therapy.[6-8] One possible explanation for this discrepancy in results is the difference in methods. Several methods used for the determination of hyaluronic acid concentration by others[1, 3-9] were tried extensively and found unsatisfactory in our hands before the method used in this study was tried and found to be reliable and reasonably accurate. In addition, other investigators have used various formulas to relate relative viscosity to concentration of hyaluronic acid as a means of studying polymerization, rather than the direct measurement of intrinsic viscosity by means of a standard plot as was used in this study. A different approach, the measurement of anomalous viscosity, used by Sundblad and associates,[9] has demonstrated an apparent increase in degree of polymerization after steroid therapy of rheumatoid arthritis, averaging 63 per cent. This method does not depend on a measurement of concentration and measures the fraction of hyaluronic acid which is most highly polymerized, rather than the mean degree of polymerization which is reflected by the intrinsic viscosity. Their method is, therefore, potentially more informative, and our data on the effect of steroid therapy should be evaluated with their results in mind.

SUMMARY

The concentration and intrinsic viscosity of hyaluronic acid in knee synovial fluid was measured in normals and a variety of types of arthritis. The intrinsic viscosity was found to be significantly lowered in 80 per cent of a group of patients with rheumatoid arthritis and in many patients with other joint diseases. No changes specific for any disease were found.

In patients with rheumatoid arthritis, there was no correlation between the degree of lowering of the intrinsic viscosity and the clinical aspects of the disease.

No correlation was found between the lowering of the intrinsic viscosity and the severity of the inflammation of the joint.

Minimal changes in intrinsic viscosity were observed after steroid therapy of patients with rheumatoid arthritis.

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LEVELS OF HEXOSAMINE AND HEXOSE IN PLASMA AND URINE IN PATIENTS WITH CERTAIN DISEASES OR INJURIES

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INTRODUCTION

ELEVATIONS of protein-bound carbohydrate material in plasma commonly occur in the course of many diseases,[1] in trauma,[2, 3] and burns.[3, 4] Recently the levels of mucoprotein components in the dialyzed urine of normal males were reported.[5] It was thought of interest to compare the levels of some high molecular weight carbohydrate components in the plasma and urine of a few patients suffering from certain diseases or from acute trauma or burns. Much of the urinary hexosamine is of low molecular weight and freely dialyzable,[5] and many previous studies[6-9] have been concerned with the total rather than with the high molecular weight hexosamine-containing material in urine.

MATERIALS AND METHODS

Nine patients, three of whom were female, were followed on the wards of Fitzsimons Hospital. Blood and urine samples were obtained soon after admission of the patients (*I*, Table I). Where possible, a second sample of blood and urine was obtained at some later date in the course of the patients' illness, or after clinical recovery (*II*, Table I).

Urine.—Twenty-four-hour urine collections were made in polyethylene bottles with added thymol. Analyses of total hexosamine and hexose were made on dialyzed aliquots, as described previously.[5]

Plasma.—About 10 ml. of venous blood was drawn from each patient at some time during the course of the 24-hour urine collection period and added to 0.2 ml. of oxalate solution.† No attempt was made to collect fasting blood samples.[3,10] The blood was centrifuged at once and determinations were made on the plasma in duplicate. Hexosamine and hexose were determined on the ethanolic precipitate of plasma (total glycoprotein fraction) and on the perchloric acid soluble, phosphotungstic acid insoluble precipitate of plasma (seromucoid fraction[1, 11]). These methods are described in detail elsewhere.[1, 12] Normal values were obtained from a small group of laboratory personnel. The hexosamine values are slightly lower and the hexose figures (as galactose-mannose) slightly higher than the most recently reported data of Winzler.[1]

RESULTS

Table I summarizes the levels of hexosamine and hexose found in the total glycoprotein and the seromucoid fractions of plasma and in dialyzed urine of

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†Four per cent (w/v) potassium oxalate, 6 per cent (w/v) ammonium oxalate in distilled water.

TABLE I. LEVELS OF HEXOSAMINE AND HEXOSE IN PLASMA AND DIALYZED URINE IN PATIENTS WITH CERTAIN DISEASES OR INJURIES

	PLASMA (MG. %)				URINE (MG./24 HRS.)		
	GLYCOPROTIEN		SEROMUCOID				
	HEXOSAMINE	HEXOSE	HEXOSAMINE	HEXOSE	HEXOSAMINE	HEXOSE	DIAGNOSIS
Normal	73.3 ± 6.0*	134 ± 18.0	7.3 ± 1.7	9.4 ± 1.8	28.9 ± 6.3	38.4 ± 9.0	
Gr I	109	214	15.5	15.2	45.7	74.7	I. Acute rheumatic fever with carditis polyarthritis. II. 3 weeks later, negative physical, asymptomatic
Gr II	71.4	152	6.1	19.7	17.5	36.1	
Ot I	90.6	176	11.3	11.6	52.5	49.1	I. Acute rheumatic fever with polyarthritis. II. 5 weeks later asymptomatic
Ot II	–	–	–	–	19.0	26.3	
Tr I	89.3	184	11.9	11.5	36.6	61.2	I. Acute rheumatic fever with carditis, polyarthritis. II. 1 month later, asymptomatic, negative physical
Tr II	83.0	172	8.7	10.1	24.5	49.7	
Ro (♀)	126	329	23.2	25.7	24.5	51.9	Acute exacerbation of chronic rheumatoid arthritis
Ch I	91.6	244	15.1	23.2	72.9	89.9	I. Acute leukemia, 1 month's duration. II. 2 weeks later, on ACTH, progressive downhill course
Ch II	100	266	15.1	17.0	101	–	
An (♀)	95.6	–	12.8	–	99.7	154	Lupus erythematosus with nephrotic syndrome
Ja I	70.2	184	–	–	165	881	I. Burn — 57% body surface, 30% 3rd degree, 4 days. II. 28 days later preterminal
Ja II	86.3	179	21.6	19.5	178	161	
Wo I (♀)	86.5	196	28.0	27.0	75.9	78.9	I. Multiple, comminuted fractures, 3 days postarthrotomy and debridement. II. 9 days later
Wo I	78.4	194	17.2	18.4	39.4	54.1	
Ed	91.0	1,250	9.9	436	64.7/ 10 hrs.	320/ 10 hrs.	Severe head injury, left hemiparesis

*Standard deviation.

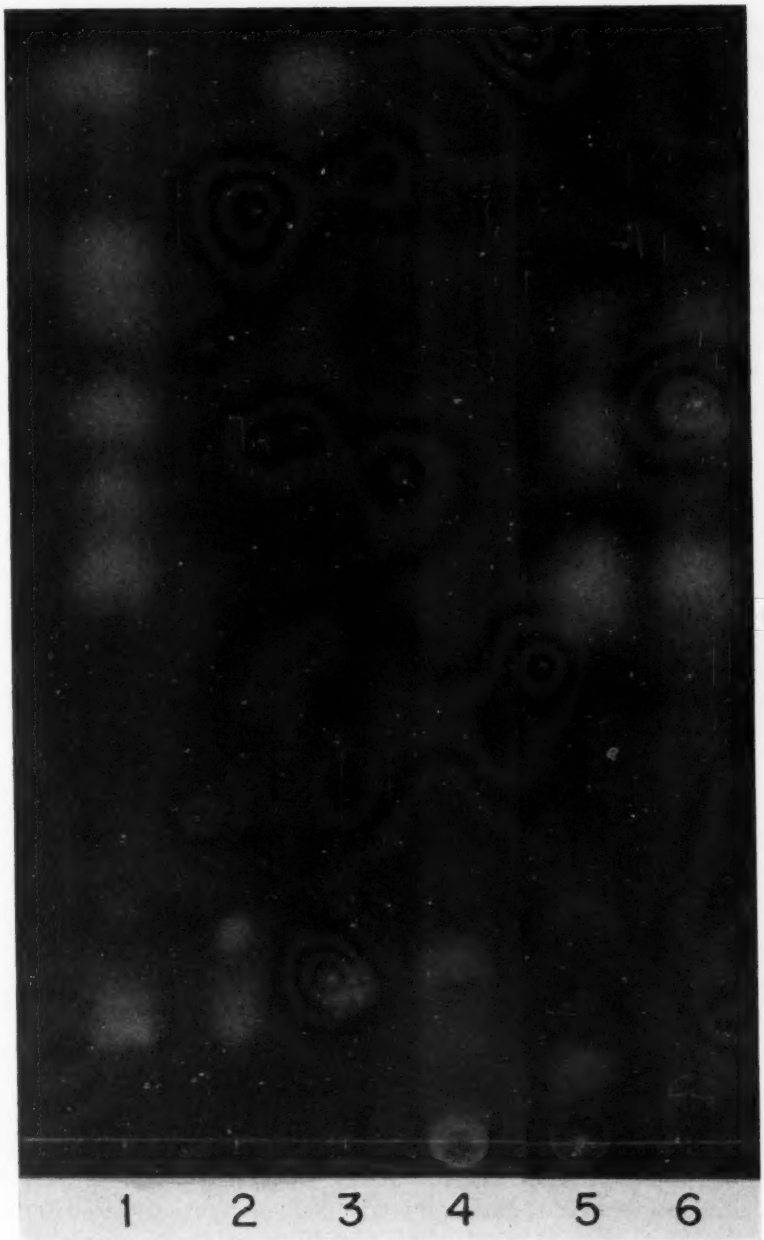

Fig. 1.—Unidimensional ascending chromatogram in 1-butanol, pyridine, and water (3:2:1.5) of standard substances and hydrolyzed nondialyzable urine solids of a patient with acute rheumatic fever (Gr). *1*, From the top down: glucuronolactone, ribose, fucose, mannose glucose, galactose, and glucuronic and galacturonic acids. The latter two substances are not separated. *2*, Galacturonic acid; *3*, glucuronic acid; *4*, hydrolyzed heparin; *5*, sample Gr I, from the top down: fucose, mannose, galactose, and an unidentified component, staining pink with β-naphthylamine, as did standard hexuronic acids, but migrating very slightly faster than these substances. *6*, Gr II: similar sugars, without component in sample I.

the subjects studied. Proteinuria, as detected by the heat and acetic acid test, was found only in Patients An and Ja I. Elevation of plasma levels of hexosamine and hexose early in the course of the patients' illness was accompanied by significant increases in one or both of these components in dialyzed urine. In those patients studied who showed clinical improvement, a fall in plasma levels was accompanied by return of urinary values to normal. Two patients suffering from trauma and one severely burned individual were also studied. The elevation of plasma hexosamine and hexose, particularly in the seromucoid fractions of these injured patients, are to be noted. Urinary levels generally exceeded the highest values found during an acute illness. The enormous value for urinary hexose in Ja I is attributed to excretion of dextran, since this polysaccharide was administered to the patient just prior to the first urine collection. Chromatography of hydrolyzed nondialyzable solids[13] prepared from the urine of this patient revealed a spot with an R_f identical to glucose. A strikingly high plasma and urinary hexose value was found in Patient Ed. This cannot be attributed to the administration of high molecular weight carbohydrate material. The nature of the hexose has not been determined.

Chromatography of hydrolyzed, nondialyzable solids was carried out to determine if the carbohydrate components of pathologic urine were similar to those observed in normal urine.[13] In the cases studied, fucose, mannose, and galactose were found, as in normal material. In Patient Gr with acute rheumatic fever, chromatograms of urine I revealed a spot staining pink with β-naphthylamine which corresponded in staining reaction but migrated at a very slightly faster rate than standard hexuronic acids (glucuronic and galacturonic acids). This spot was not present in the recovery urine II (Fig. 1).

DISCUSSION

Increased urinary levels of material containing carbohydrate and nitrogen, both of high and low molecular weight, have been reported in many diseases not primarily involving the kidneys as well as in calculous disease. Increased excretion of a "proteose" in the urine of patients suffering from acute allergic states, phthisis, and "rheumatism" was noted over 20 years ago by Oriel.[14, 15] More recently Taylor and associates[16] found increased high molecular weight material containing nitrogen in the blood and urine of burned patients. Increased carbohydrate material in urine as measured by the Donaggio reaction was described during "stress," and was partly characterized as a mucoprotein.[17, 18] Increases in urinary hexosamine in diabetes[6] or in "collagen diseases"[7, 8] was reported in undialyzed urine. No consistent elevations of a nondialyzable hexuronic acid containing component of urine, isolated by benzidine precipitation, was observed in diabetics without renal impairment by Craddock and Kerby.[19] A number of quantitative studies have been made recently by Boyce and associates[20, 21] on biocolloids of urine in health and calculous disease. In urine of patients with renal calculi, these workers found considerable increases in the dry weight of material insoluble in molar sodium chloride which appears to be largely mucoprotein. Boas and associates[9] have measured plasma and urinary hexosamine in a variety of diseases. To avoid interfering effects of urinary chromogens in the analytic determinations, they isolated hexosamine

from hydrolysates of undialyzed urine on Dowex-50 resin. By these methods, as would be expected, they obtained a mean normal value of 84 mg. of hexosamine per 24 hours, a value three times that found by us using dialyzed urine.[5] This total urinary hexosamine was found to be increased in a number of cases of acute clinical stress associated with elevations of total plasma hexosamine.

The studies reported in this paper have shown that elevations of plasma protein-bound hexosamine and hexose in a number of diseases are associated with increased excretion of one or both of these substances in dialyzed urine. In three patients with traumatic injuries or burns, marked increases in urinary excretion of nondialyzable hexose and hexosamine occurred. With the exception of a patient (An) with the nephrotic syndrome secondary to lupus erythematosus, the kidneys were not directly involved by the disease or injury. Proteinuria as revealed by the heat and acetic acid test was not found except in Patient An and in the first urine sample of a burned patient (Ja I).

These studies do not clarify the question of origin of the increased carbohydrate material in the urine described in this paper. There is very little information to date on either the chemical identity or origin of mucoproteins or mucopolysaccharides in urine. Kerby[22] demonstrated a nondialyzable hexuronic acid containing component in normal urine which on paper chromatography migrated at a rate similar to chondroitin sulfate and speculated on the origin of this material from the blood by glomerular filtration. The only homogeneous mucoprotein fraction isolated from the urine thus far[23] is of enormous molecular weight[24] and presumably arises from the urinary tract itself. This mucoprotein appears to be increased in subjects with local injury to the genitourinary tract, as in pyelonephritis and calculous disease, but not in patients with systemic disorders such as lupus erythematosus or multiple myeloma.[25] In diseases such as these, increases in mucoproteins in the urine may be ascribed to the same renal alterations that lead to the loss of plasma protein. Increased mucoprotein components in urine during the course of disease or injury not directly involving the kidneys are associated with increased plasma levels of these substances, and it seems possible that they could be derived from the blood by glomerular filtration. The excretion of high molecular weight material present in the blood may partly depend on blood levels, glomerular permeability, tubular reabsorption of the material, urine flow, and molecular size. In regard to this last factor, it is of interest that alterations of molecular size may come about during the course of disease. Crossley and associates[26] have shown in vivo breakdown of serum proteins to molecules the size of proteoses and peptones during pneumococcus infection.

SUMMARY

Hexosamine and hexose have been measured in the glycoprotein and seromucoid fraction of plasma and in the dialyzed urine of patients with certain diseases or acute injury not directly involving the kidney. Elevations of plasma levels were associated with increases in one or both of these components in dialyzed urine.

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INTRINSIC FACTOR STUDIES

IV. Selective Absorption and Binding of Cyanocobalamin by Gastric Juice in the Presence of Excess Pseudovitamin B_{12} or 5,6-dimethylbenzimidazole

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THE intrinsic factor in normal human gastric juice enhances the absorption of cyanocobalamin in patients with pernicious anemia. Such gastric juice also binds cyanocobalamin as estimated by microbiologic assay,[1] dialysis,[2] or paper electrophoresis.[3] The fact that binding of this vitamin is not equivalent to intrinsic factor activity is apparent from several reports.[4–6] However, as far as can be determined from published data, all intrinsic factor preparations bind some vitamin B_{12}. Bishop, Toporek, Nelson, and Bethell[7] have interpreted their recent observations as demonstrating that vitamin B_{12} already bound to intrinsic factor is absorbed preferentially over other added B_{12} They concluded that vitamin B_{12} binding power is necessary for intrinsic factor activity. Recent studies in this laboratory, prompted by their report, have confirmed their findings.[8] We are not aware of data supporting the recent statement, "It appears that vitamin B_{12} binding is not a property of intrinsic factor."[9]

One moiety contributing to the structure of the cyanocobalamin molecule is 5,6-dimethylbenzimidazole.[10] When this portion is replaced by adenine the compound is known as pseudovitamin B_{12}.[11, 12]† That the latter substance supports growth of some microbes but cannot be substituted for B_{12} in chicks was noted by Pfiffner and co-workers.[13] Firth and Johnson[14] concluded that pseudo-B_{12} is inactive for baby pigs. It has been reported that pseudo-B_{12} lacks antipernicious anemia activity.[15] Ford, Holdsworth, and Porter[16] observed that the pseudovitamin is abundant in cow rumen and pig cecal contents but absent from the livers of these animals. The possibility that the absence of pseudo-B_{12} in tissues results from decreased absorption or preferential urinary excretion is being investigated by them.

It was of interest to determine whether pseudo-B_{12} competes with B_{12} for the mechanism which effects intestinal absorption of B_{12}. In the present report the effect of excess pseudo-B_{12} or DMBI upon the in vivo absorption of $B_{12}Co^{60}$ was studied. The effect of these two compounds upon the in vitro

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†Hereafter, the following abbreviations will be used: NHGJ = Normal human gastric juice; B_{12} = cyanocobalamin (i.e., vitamin B_{12}); $B_{12}Co^{60}$ = radioactive cyanocobalamin; DMBI = 5,6-dimethylbenzimidazole; pseudo-B_{12} = pseudovitamin B_{12}.

binding of $B_{12}Co^{60}$ by gastric juice or serum was studied by dialysis.[2] By a combination of these two methods intrinsic factor activity and certain in vitro binding properties of gastric juice were correlated. Finally, the effectiveness of pseudo-B_{12} as a flushing agent for absorbed $B_{12}Co^{60}$ was studied.

MATERIALS AND METHODS

Absorption.—The urine radioactivity technique[17, 18] was utilized for estimating absorption of orally administered $B_{12}Co^{60}$.* Using simultaneous urine and fecal radioactivity techniques, Callender and Evans[19] showed that urine radioactivity is proportional to the amount of $B_{12}Co^{60}$ absorbed. The crystalline $B_{12}Co^{60}$ preparation used in the present study contained 95 per cent cyanocobalamin and 3 per cent hydroxycobalamin[20]; its specific activity was adjusted to about 250 μc per milligram by dilution with non-radioactive B_{12}. In some instances, excess B_{12}, DMBI†, or crystalline pseudo-B_{12}‡ was included in the oral test material. It is known that the pseudovitamin preparation is entirely free of B_{12} and DMBI.[21] Patients who lacked intrinsic factor were given an aliquot from a pool of neutralized NHGJ simultaneously with $B_{12}Co^{60}$ by mouth to insure absorption. Pools of lyophilized or frozen NHGJ were prepared as described previously.[3] Solutions of crystalline B_{12}, "Redisol"§ or "Dodex" (Organon), constituted the 1,000 μg flushing dose injected two hours after the oral administration of $B_{12}Co^{60}$.

Dialysis.—Rosenblum, Woodbury, and Reisner[22] have found the combination of tracer vitamin and dialysis techniques to be suitable for measuring in vitro binding of vitamin B_{12}; in their hands it proved to be a simple and highly reproducible method. With use of a slightly modified techinque developed by one of us,[2] the above advantages were again noted. Thus, dialysis was chosen to measure in vitro binding of $B_{12}Co^{60}$ by gastric juice or serum. The method is as follows: (1) addition of an aliquot of biologic material to known amounts of $B_{12}Co^{60}$ and other test substances; (2) equilibration at room temperature for one hour; (3) determination of radioactivity (X) in a well-type scintillation counter; (4) exhaustive dialysis of the radioactive mixture confined in tubes of Visking casing suspended in running cold tap water for about 60 hours; and (5) examination of the dialyzed sample for radioactivity (Y). Then $\frac{Y}{X} \times 100 =$ per cent retention B_{12}. The concentration of bound vitamin may be calculated from this. When a mixture of $B_{12}Co^{60}$ and water is dialyzed under the same conditions, the radioactivity remaining within the dialysis bag is less than 1 per cent. When aliquots of serum were dialyzed, Veronal buffer (pH 8.6), changed every 2 or 3 hours except evernight, replaced running water. Some of the NHGJ samples examined by this method were aliquots from the pools administered orally to patients.

Paper Electrophoresis.—Binding of $B_{12}Co^{60}$ by an intrinsic factor concentrate in vitro was determined by a paper electrophoresis technique described previously.[3] This intrinsic factor concentrate, UFR,‖ has been reported by Williams and colleagues[9] to be clinically active in a daily dose of 1 or 2 mg. with 10 μg of B_{12}.

In Vivo Binding.—A weighed crystalline preparation of pseudo-B_{12} was diluted in saline to a concentration of 100 μg per milliliter. With use of the published extinction values at 361 mμ for pseudo-B_{12},[23] the concentration was determined spectrophotometrically and found to be 122 μg per milliliter. A commercial solution of crystalline B_{12} labeled 1,000 μg per milliliter was diluted with saline to give a concentration of 100 μg per milliliter. The concentration of this dilution determined spectrophotometrically was 122 μg per milliliter. The urinary excretion of microbiologic "vitamin B_{12} activity" in the 24 hours following injection was estimated with *Lactobacillus leichmannii* in an 18-hour

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§Obtained through the courtesy of N. Ritter, Merck & Company.

‖Kindly furnished by W. L. Williams, Lederle Laboratories, Pearl River, N. Y.

turbidimetric assay.* Appropriate dilutions of the injected materials were also assayed and the per cent of the injected dose excreted in the urine was calculated. It should be noted that 122 μg of pseudo-B_{12} provided a growth response in this microbiologic assay equal to 67 μg of U.S.P. B_{12} reference standard. This magnitude of activity for *L. leichmannii* is in agreement with published figures.[24, 25] The 122 μg of B_{12} resulted in a growth response equal to 133 μg of the U.S.P. reference standard. One milliliter of either solution was injected at 48-hour intervals into 2 patients. (See Table VI for protocol.)

RESULTS

Absorption.—The effect of excess B_{12}, pseudo-B_{12}, or DMBI upon the absorption of orally administered $B_{12}Co^{60}$ was studied in 6 patients who were known to lack intrinsic factor. Each subject served as his own control by means of repeated observations, as noted in Table I. Adding a fifty-fold excess of B_{12} to the oral drink of $B_{12}Co^{60}$ and gastric juice caused a marked diminution in urinary radioactivity. Pseudo-B_{12} or DMBI exerted no such effect.

Next tested was the effectiveness of parenterally administered pseudo-B_{12} as an agent for flushing absorbed $B_{12}Co^{60}$ into the urine. Data in Table II show that urine radioactivity was definitely less when pseudo-B_{12} was used as a flushing agent. The lower excretion values represent some flushing activity by the pseudovitamin, because when no flushing agent is given no radioactivity appeared in the urine (see *observation 3,* AW, Table II). Injection of 1,000 μg B_{12} into this patient at 24 hours did flush 7.6 per cent of ingested radioactivity into the 24- to 48-hour urine sample.

Dialysis. A. *Gastric Juice.*—The effect of excess B_{12}, pseudo-B_{12}, or DMBI upon the binding of $B_{12}Co^{60}$ by 4 different pools of neutralized NHGJ was studied. It had been determined previously that when as many as 150 mμg B_{12} were added per milliliter NHGJ, 40 mμg per milliliter remained bound after dialysis.[2] It was decided, therefore, to use in these studies a concentration of 50 mμg $B_{12}Co^{60}$ per milliliter. Representative findings, listed in Table III, indicate that pseudo-B_{12} is a very ineffective competitor for the B_{12} binding sites of NHGJ. At higher concentrations of $B_{12}Co^{60}$ and pseudo-B_{12} it was noted that pseudo-B_{12} offered very slight competition for the B_{12} binding sites of pool 12 NHGJ. In all cases, 50x B_{12} caused a marked decrease in $B_{12}Co^{60}$ binding. Binding of $B_{12}Co^{60}$ was not reduced by the presence of excess DMBI. Pseudo-B_{12} was not a more effective competitor for binding sites in the presence of DMBI.

The data recorded in the same table confirm earlier observations of $B_{12}Co^{60}$ binding capacity of NHGJ (44 mμg/ml. when $B_{12}Co^{60}$ was added at a level of 50 mμg/ml. NHGJ). The B_{12} binding capacity of NHGJ rises slightly when excessively large amounts of the vitamin are present (70 mμg/ml. when $B_{12}Co^{60}$ is added at a level of 2,550 mμg/ml. NHGJ).

The following experiments were to determine the effect of boiling upon the ability of neutralized NHGJ to bind $B_{12}Co^{60}$ in the presence of excess B_{12} or pseudo-B_{12}. It had been found earlier that boiling NHGJ lowers its

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TABLE I. RADIOACTIVITY EXCRETED FOLLOWING ORAL ADMINISTRATION OF $B_{12}CO^{60}$ ALONE AND IN COMBINATION WITH B_{12}, PSEUDO-B_{12}, OR DMBI

PATIENT AND DIAGNOSIS	OBSERVATIONS	ORALLY ADMINISTERED SUBSTANCES*						% RADIOACTIVITY IN 24-HR. URINE
		NHGJ† POOL	ML.	1X $B_{12}CO^{60}$ (1 μG)	50X B_{12} (50 μG)	50X PSEUDO B_{12} (50 μG)	50X DMBI (5 μG)‡	
MN (PA)	1	13	50	+	−	−	−	12.0
	2	13	50	+	+	−	−	0.8
	3	13	50	+	−	+	−	11.0
AM (PA)	1	1	50	+	−	−	−	13.0
	2	13	25	+	+	−	−	0.0
	3	13	25	+	−	+	−	17.0
MW (PA)	1	13	50	+	−	−	−	6.4
	2	13	50	+	+	−	−	0.0
	3	13	50	+	−	+	−	4.4
CA (TG)	1	10	50	+	−	−	−	13.0
	2	14	25	+	−	−	+	13.0
MN (PA)	1	14	25	+	−	−	−	7.4
	2	14	25	+	−	−	+	10.0
ME (Sub-TG)	1	14	25	+	−	−	−	9.3
	2	14	25	+	−	−	+	8.1

PA = Pernicious anemia. TG = Total gastrectomy.

*Each patient received 1,000 μg crystalline B_{12} subcutaneously 2 hours after the orally administered substances.

†NHGJ aliquot was added to $B_{12}Co^{60}$ or to the mixture of $B_{12}Co^{60}$ and B_{12}, pseudo-B_{12}, or DMBI.

‡ Molar equivalent of 50 μg B_{12} (Mol. wt. DMBI = 146).

TABLE II. COMPARISON OF B_{12} AND PSEUDO-B_{12} AS FLUSHING AGENTS IN THE URINE RADIOACTIVITY TECHNIQUE

PATIENT AND DIAGNOSIS	OBSERVATION	ORAL DOSE: 1 μG $B_{12}CO^{60}$	FLUSHING DOSE: 1,000 μG*		% RADIOACTIVITY IN 24-HR. URINE
			B_{12}	PSEUDO-B_{12}	
VB (Control)	1	+	−	+	7.7
	2	+	+	−	26.0
WK (Control)	1	+	−	+	3.8
	2	+	+	−	17.0
GR (Control)	1	+	−	+	8.8
	2	+	+	−	21.0
FG (PA)	1	+	−	+	6.2
	2	+	+	−	10.0
AW (Control)†	1	+	+	−	12.0
	2	+	−	+	2.9
	3	+	−	−	0.0‡
PS (PA)†	1	+	+	−	11.0
	2	+	−	+	5.3

PA = Pernicious anemia.

*Injected subcutaneously 2 hours after oral $B_{12}Co^{60}$.

†Note that in these patients the sequence of administering flushing agents was reversed.

‡Injection of 1,000 μg B_{12} at 24 hours did flush 7.6 per cent of injected radioactivity into the 24- to 48-hour urine sample.

TABLE III. EFFECT OF EXCESS B_{12}, PSEUDO-B_{12}, OR DMBI ON THE BINDING OF $B_{12}Co^{60}$ BY HUMAN GASTRIC JUICE

NHGJ POOL*	ALIQUOT NO.	1x $B_{12}CO^{60}$ (50 mμG/ML.)	50x B_{12} (2,500 mμG/ML.)	50x PSEUDO-B_{12} (2,500 mμG/ML.)	50x DMBI (260 mμG/ML.)†	% RETENTION $B_{12}CO^{60}$ AFTER DIALYSIS
12	1	+	−	−	−	91.0
	2	+	+	−	−	3.3
	3	+	−	+	−	94.0
13	1	+	−	−	−	88.0
	2	+	+	−	−	2.9
	3	+	−	+	−	76.0
13a	1	+	−	−	−	86.0
	2	+	+	−	−	3.5
	3	+	−	−	+	92.0
14	1	+	−	−	−	76.0
	2	+	+	−	−	3.1
	3	+	−	+	−	55.0
	4	+	−	−	+	77.0
	5	+	−	+	+	56.0

*The NHGJ aliquot was added to $B_{12}Co^{60}$ or to the mixture of $B_{12}Co^{60}$ and B_{12}, pseudo-B_{12}, or DMBI.

†Molar equivalent of 2,500 mμg B_{12} per milliliter.

B_{12} binding capacity. Therefore, the concentration of $B_{12}Co^{60}$ was reduced from 50 mμg/ml. to 10 mμg/ml. Excess pseudo-B_{12} did not alter the per cent retention of $B_{12}Co^{60}$ by nonboiled NHGJ (*aliquot 5*, Table IV). However, the amount of $B_{12}Co^{60}$ retained by boiled NHGJ in the presence of excess pseudo-B_{12} was lowered. An additional observation confirmed this.

TABLE IV. EFFECT OF BOILING NEUTRALIZED GASTRIC JUICE UPON ITS ABILITY TO BIND $B_{12}Co^{60}$ IN THE PRESENCE OF EXCESS B_{12} OR PSEUDO-B_{12}

NHGJ POOL 12*	ALIQUOT NO.	1x $B_{12}CO^{60}$ (10 mμG/ML.)	50x B_{12} (500 mμG/ML.)	50x PSEUDO-B_{12} (500 mμG/ML.)	% RETENTION $B_{12}CO^{60}$ AFTER DIALYSIS
UNB	1	+	−	−	87.0
B	2	+	−	−	89.0
UNB	3	+	+	−	6.7
B	4	+	+	−	5.0
UNB	5	+	−	+	86.0
B	6	+	−	+	65.0

UNB = Unboiled. B = Boiled.

*The NHGJ aliquot was added to $B_{12}Co^{60}$ or to the mixture of $B_{12}Co^{60}$ and B_{12} or pseudo-B_{12}.

B. *Serum.*—Either 1 or 2 mμg $B_{12}Co^{60}$ was added to 1 ml. aliquots of human serum. When larger quantities of the vitamin are added to serum, the per cent retention decreases but the quantity bound increases (Table V). As would be expected, addition of 50x molecules of B_{12} reduced retention of $B_{12}Co^{60}$ markedly. The same result was observed when 50x pseudo-B_{12} was substituted for B_{12}. However, 50x DMBI did not reduce binding of $B_{12}Co^{60}$ by serum.

TABLE V. EFFECT OF EXCESS B_{12} PSEUDO-B_{12} OR DMBI ON THE BINDING OF $B_{12}Co^{60}$ BY HUMAN SERUM

SERUM POOL*	ALIQUOT NO.	1x $B_{12}CO^{60}$ (1 MμG/ML.)	50x B_{12} (50 MμG/ML.)	50x PSEUDO-B_{12} (50 MμG/ML.)	50x DMBI	% RETENTION $B_{12}CO^{60}$ AFTER DIALYSIS
MB	1	+	–	–	–	85.0
	2	+	+	–	–	15.0
	3	+	–	+	–	19.0
KS	1	+	–	–	–	82.0
	2	+	+	–	–	15.0
	3	+	–	+	–	18.0
		(2 MμG/ML.)	(100 MμG/ML.)	(100 MμG/ML.)	(10 MμG/ML.)†	
MB	1	+	–	–	–	49.0‡
	2	+	+	–	–	6.3
	3	+	–	+	–	9.7
	4	+	–	–	+	41.0
	5	+	–	+	+	7.3

*The serum aliquot was added to $B_{12}Co^{60}$ or to the mixture of $B_{12}Co^{60}$ and B_{12}, pseudo-B_{12}, or DMBI.

†Molar equivalent of 100 mμg B_{12} per milliliter.

‡Per cent retention is lower because binding capacity for B_{12} has been exceeded.

Paper Electrophoresis.—B_{12} binding by an intrinsic factor concentrate, UFR,[9] was investigated. It is apparent from Fig. 1 that all $B_{12}Co^{60}$ was bound. No radioactivity was detected 2 or 3 cm. toward the cathode, the area where unbound $B_{12}Co^{60}$ would be located. The peak of radioactivity was present 5 cm. from the origin towards the anode. When a $B_{12}Co^{60}$-NHGJ mixture was electrophoresed in the same manner, the largest amount of radioactivity was also present 5 cm. from the origin. Not all the added $B_{12}Co^{60}$ was bound by gastric juice. The NHGJ sample used was known to be a potent promoter of $B_{12}Co^{60}$ absorption in several pernicious anemia patients.

TABLE VI. URINARY EXCRETION OF MICROBIOLOGIC B_{12} ACTIVITY FOR 24 HOURS AFTER INTRAMUSCULAR INJECTION OF PSEUDO-B_{12} OR B_{12}

PATIENT	TIME IN HOURS	INJECTION (122 μG) PSEUDO-B_{12}	INJECTION (122 μG) B_{12}	MICROBIOLOGIC B_{12} ACTIVITY (PSEUDO-B_{12} + B_{12}) μG/24 HR. URINE	% INJECTED DOSE IN 24-HR. URINE*
WA	0-24 (Control)	–	–	0.1	
	24	+	–	---	
	24-48	–	–	17.0	25
	72-96 (Control)	–	–	0.3	
	96	–	+	---	
	96-120	–	–	46.6	35
WH	0-24 (Control)	–	–	0.1	
	24	+	–	---	
	24-48	–	–	24.7	37
	72-96 (Control)	–	–	0.4	
	96	–	+	---	
	96-120	–	–	57.4	43

*The injected and excreted quantities as determined by microbiologic assay were used for calculating this percentage.

In Vivo Binding.—Data presented in Table VI demonstrate that similar amounts of B_{12} or pseudo-B_{12} are excreted in the urine 24 hours after parenteral injection of 122 μg.

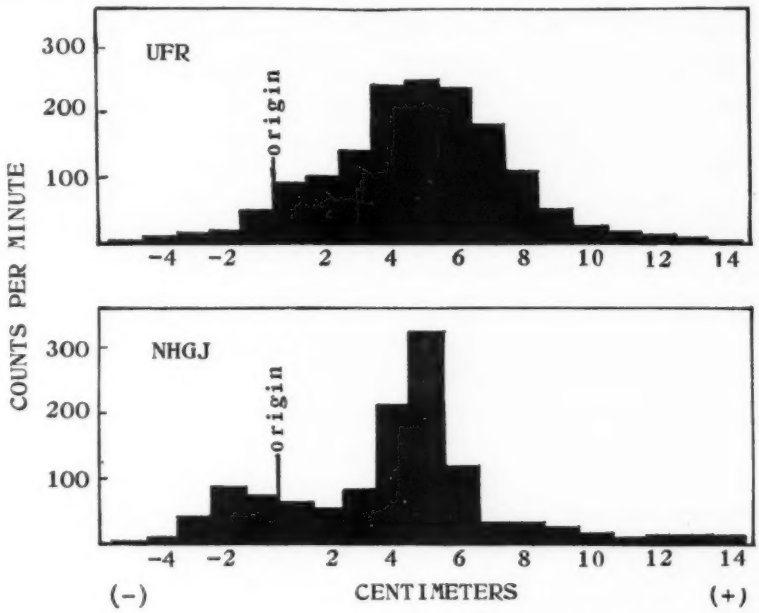

Fig. 1.—Localization of radioactivity after paper electrophoresis of 5.9 mμg $B_{12}Co^{60}$ mixed with 75 μg intrinsic factor concentrate (UFR) or nondialyzable NHGJ solids (Veronal buffer pH 8.6).

DISCUSSION

Throughout this report it is assumed that $B_{12}Co^{60}$ and B_{12} are biologically identical. It is apparent from Table I that B_{12} is in competition with $B_{12}Co^{60}$ for absorption, because less radioactivity appears in the urine when a fifty-fold excess of B_{12} is given orally with $B_{12}Co^{60}$. This result would be anticipated on the basis of Glass's documented postulate of decreasing efficiency of absorption with increasing oral intake of B_{12}.[26] The data in Table I show that neither pseudo-B_{12} nor DMBI is in competition with $B_{12}Co^{60}$ for the physiologic mechanism effecting absorption of the latter. It is interesting that man does not utilize the B_{12} absorptive mechanism to absorb pseudo-B_{12}, a form of the vitamin which lacks hemopoietic activity[15] and presumably other biologic functions for man. The data in Table I provide no evidence for a lack of absorption of pseudo-B_{12}.

The data in Table II indicate that parenterally injected pseudo-B_{12} is not as effective as B_{12} as a flushing agent for absorbed $B_{12}Co^{60}$. The retention of pseudo-B_{12} after subcutaneous injection is similar to that of B_{12} (Table VI). In vitro experiments indicate that pseudo-B_{12} is as effective a competitor for serum $B_{12}Co^{60}$ binding sites as is B_{12} itself. Why then is pseudo-B_{12} not as effective a flushing agent? Our data provide no clues to the answer. Perhaps B_{12} displaces some absorbed $B_{12}Co^{60}$ which has been bound to tissues other

than serum and the pseudo-B_{12} is unable to effect this displacement. To test the in vivo binding of pseudo-B_{12} and B_{12} it was assumed that urinary excretion of parenterally injected pseudo-B_{12} or B_{12} was inversely related to such binding. On the basis of this assumption, the data in Table VI indicate that in vivo binding of pseudo-B_{12} and B_{12} are roughly equivalent.

The binding of B_{12} by NHGJ is a highly selective process as evidenced by the fact that so minor a change as substituting adenine for DMBI in the vitamin B_{12} molecule leads to a failure of the new product, pseudo-B_{12}, to compete with B_{12} for the mechanism of B_{12} binding. Because the Co^{60} indicator was always part of the B_{12} molecule, the data in Table III do not indicate failure to bind pseudo-B_{12}. In one of the pools there was slight competition between B_{12} and pseudo-B_{12} for binding sites. This is interpreted as evidence that some NHGJ preparations have nonspecific as well as specific cobalamin binding mechanisms. Boiling destroys many B_{12} binding sites[2] and renders others nonspecific (Table IV).

Using dialysis and growth inhibition techniques described by Bird and Hoevet,[27] Bird[28] found that a crude intrinsic factor preparation bound the same amount of pseudo-B_{12} as B_{12}. Since these observations were not testing competitive binding, they are not in conflict with the selective $B_{12}Co^{60}$ binding observed by us in NHGJ. Gregory and Holdsworth[29] have evidence that B_{12} is preferentially bound by an intrinsic factor concentrate exposed to a mixture of B_{12}, pseudo-B_{12}, and Factors A and B.

Rosenblum and co-workers[22] found that intrinsic factor preparations bind more chlorocobalamin than B_{12}. No evidence is available to indicate whether these two forms of the vitamin are in competition for the same binding sites in NHGJ. In subsequent reports[30, 31] it was noted that normal subjects absorbed less chloro-, sulfato-, nitro-, or thiocyanatocobalamin than cyanocobalamin.

It is believed that the data cited in Tables III and V indicate a fundamental difference in the type of B_{12} binding occurring in NHGJ as compared to serum. Data in Tables I and III indicate a correlation between selective in vitro binding by NHGJ and in vivo absorption of B_{12}. These observations are in accord with the recently rejuvenated theory[7] that B_{12} binding is a necessary property of intrinsic factor. As noted in the introduction, published data show that all intrinsic factor preparations bind B_{12}. That selective binding of B_{12} is a necessary but not sufficient attribute for intrinsic factor activity is indicated by the finding that boiled NHGJ retains some selective binding for B_{12} (Table IV) but lacks intrinsic factor activity. The preparation of pure intrinsic factor will make it possible to determine if binding is in stoichiometric relationship to intrinsic factor activity.

SUMMARY AND CONCLUSIONS

1. Neither pseudovitamin B_{12} nor 5,6-dimethylbenzimidazole competes with radioactive cyanocobalamin for the physiologic mechanisms effecting absorption of cyanocobalamin.

2. Pseudovitamin B_{12} is decidedly less effective than cyanocobalamin as a flushing agent in causing the urinary excretion of absorbed radioactive cyanocobalamin. Similar quantities of pseudovitamin B_{12} and cyanocobalamin are retained after injection of 122 μg of either.

3. The in vitro binding of cyanocobalamin by gastric juice is a selective process showing a distinct preference for cyanocobalamin over pseudovitamin B_{12} or 5,6-dimethylbenzimidazole.

4. The process of cobalamin binding by serum, unlike that in gastric juice, does not manifest a selectivity for cyanocobalamin in the presence of excess pseudovitamin B_{12}.

5. The electrophoretic mobilities of the cyanocobalamin binding substances in normal human gastric juice and in an intrinsic factor concentrate are similar.

6. The data are in accord with the hypothesis that binding of cyanocobalamin is necessary for intrinsic factor activity but binding alone is no criterion for the presence of such activity.

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STUDIES ON LEUKEMIC LEUKOCYTE EXTRACTS

I. Lack of Antigenic Relationship in Acquired Hemolytic Anemia

II. The Demonstration of a Hemolysin and Agglutinin

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HEMOLYTIC anemia which may occur in the course of chronic lymphocytic leukemia has been considered to be of an autoimmune nature. This concept has arisen because of indirect evidence of an abnormal immunologic state, and laboratory findings similar to those seen in the isoimmune hemolytic anemias. However, direct proof of such a mechanism is lacking because an antigen has not been demonstrated in spite of a search for such material in the erythrocytes and plasma. In most of the "symptomatic hemolytic anemias," abnormal cells of the lymphocytic or reticuloendothelial series are present. The possible influences of an abnormal cell type on the development of hemolytic anemia were therefore investigated. Leukemic cells were utilized and the investigation undertook to answer two questions: (1) Is material present in leukemic leukocytes which may be antigenic for the antibodies found in chronic lymphocytic leukemia complicated by hemolytic anemia? (2) Is material present in leukemic leukocytes which may directly affect erythrocytes, thereby simulating an autoimmune disease?

METHODS

A minimum of 30 ml. of blood was drawn from each patient studied and heparinized to prevent clotting. The blood was allowed to settle for a two-hour period at 6° C. and the plasma carefully pipetted from the erythrocyte layer. The erythrocytes were washed three times in large quantities of saline, and elution of coating antibodies according to the method of Kidd[1] was carried out as follows: the erythrocytes were partially lysed by an equal volume of distilled water and frozen and thawed three times. Distilled water equal to four times the original volume of erythrocytes was then added. This solution was acidified with N HCl to pH 5.6 and the precipitated stroma removed by centrifugation. The stroma was washed a minimum of eight times in large quantities of Soerensen's phosphate buffer at pH 5.6, with several interruptions for freezing and thawing. Citrate-phosphate buffer at pH 3.2 was added to the stroma in an amount equal to two volumes of the packed stroma, and the pH was adjusted to 3.2 with N HCl. This mixture was left at room temperature for twenty minutes, and the supernatant was removed after centrifugation and returned to pH 7.4 by the addition of 5 N NaOH. The resulting precipitate was discarded after centrifugation, and the supernatant containing eluted antibodies saved for testing.

The plasma was centrifuged at 3,000 r.p.m. for thirty minutes and carefully pipetted free from the leukocyte layer. The leukocyte layer was washed one time with 5 ml. of

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saline and differential cell counts done. Prolonged and rough washings were avoided in view of evidence of loss of cellular material from leukocytes during washings.[2] Cell washings were saved for testing. The leukocytes were resuspended in 0.88 M sucrose at a concentration of 2.0 ml. sucrose per 1.0 ml. packed leukocytes, and the suspension transferred to a glass homogenizer. The leukocytes were homogenized for ten minutes with the aid of a mechanical stirrer. The mixture was then centrifuged at 41,190 × g for two hours in a Spinco preparatory ultracentrifuge, and the supernatant was separated from the residual of intact cells, nuclei, mitochondria, and microsomes. It was hoped by this technique to separate soluble cytoplasmic fractions from particulate material.[3] The residual was resuspended in twice its volume of 0.14 M NaCl and homogenization was again carried out in a glass homogenizer for ten minutes. This mixture was centrifuged at 10,000 r.p.m. for forty-five minutes and the supernatant carefully separated. The residual was resuspended in twice its volume of 2.0 M NaCl and homogenization again carried out for ten minutes. The supernatant was again separated by centrifugation at 10,000 r.p.m. for forty-five minutes and the residual cellular debris discarded. The three extracts and solutions of 0.88 M sucrose and 2.0 M NaCl were dialyzed in the cold for 48 hours against repeated changes of isotonic saline. The sucrose and NaCl solutions were used as controls in all testing to eliminate the possibility of incomplete dialysis. Precipitates which formed in the dialysis bags were discarded, and the clear isotonic solutions were used for testing. The pH of all final leukocyte extract solutions were adjusted to 7.0 if they were not at this level after dialysis.

Direct and indirect antiglobulin (Coombs) tests were done in the usual manner with the following modifications: After macroscopic reading for hemolysis in the test tube, the solutions were placed on ground glass plates with polished concavity chambers and read for agglutination over indirect lighting supplied by a Quebec colony counter (Spencer). Agglutination was considered positive only when it was macroscopically visualized. All tests were checked microscopically to eliminate rouleau false positive results, and microscopic agglutination in the absence of macroscopic findings was considered ±. Group O, Rh negative erythrocytes, preserved in ACD solution (Baxter), were used for all testings, and cells older than ten days were discarded. Trypsinization was accomplished by incubating for one hour at 37° C. 0.5 ml. of thrice-washed Group O, Rh negative erythrocytes with a mixture of the following: 0.25 ml. of 1 per cent crystalline trypsin, lyophiled (Worthington) dissolved in $\frac{1}{10}$ N HCl and 2.25 ml. of Soerensen's phosphate buffer at pH 7.7.[4] The trypsinized erythrocytes were washed three times in saline prior to testing. Trypsinized erythrocyte agglutination tests (TEA) were performed with use of 0.1 ml. of test material mixed with 0.05 ml. of a 2 per cent solution of trypsinized erythrocytes suspended in saline. This mixture was incubated for three hours before being read as described for the antiglobulin tests.

Precipitin tests were performed using mixtures of erythrocyte-eluted antibodies and leukemic leukocyte extracts treated as antigens. Capillary tubes of 1.0 mm. internal diameter were filled by capillary action with equal amounts of the test solutions, embedded in a Plastalena block, and refrigerated at 6° C. for forty-eight hours before final reading.

RESULTS

Twelve patients with leukemia were studied; the diagnosis and immunologic status are summarized in Table I. Five patients had chronic leukemic lymphocytic leukemia complicated by an acquired hemolytic anemia. In four of these five cases, antibodies were recovered by eluting erythrocytes.

In Table II, the leukocyte count, volume of packed leukocytes extracted, and differential count of the extracted leukocytes are given. All patients, except Patient 4 who had a normal leukocyte count, had leukocyte cellular extract studies made.

TABLE I. THE DIAGNOSIS AND IMMUNOLOGIC STATUS OF LEUKEMIA CASES STUDIED

PATIENT	DIAGNOSIS	DIRECT COOMBS	INDIRECT COOMBS	TEA*	ELUTED ANTIBODY
1.	Chronic lymphocytic leukemia; hemolytic anemia	+	+	+	+
2.	Chronic lymphocytic leukemia; hemolytic anemia	+	+	+	+
3.	Chronic lymphocytic leukemia; hemolytic anemia	+	–	–	+
4.	Chronic lymphocytic leukemia; hemolytic anemia	+	–	–	+
5.	Chronic lymphocytic leukemia; hemolytic anemia	+	–	–	–
6.	Chronic lymphocytic leukemia	–	–	–	–
7.	Chronic lymphocytic leukemia	–	–	–	–
8.	Chronic granulocytic leukemia	–	–	+	–
9.	Chronic granulocytic leukemia	–	–	–	–
10.	Chronic granulocytic leukemia	–	–	–	–
11.	Acute granulocytic leukemia	–	–	–	–
12.	Acute monocytic leukemia	–	–	–	–

*Trypsinized erythrocyte agglutination.

Precipitin studies were done as follows: plasma from Patients 1 and 2 and erythrocyte elution solutions obtained from Patients 1, 2, 3, and 4 were considered as antibody solutions. All of these solutions were indirect Coombs positive and would agglutinate trypsinized erythrocytes. The 0.88 M sucrose, 0.14 M NaCl, and 2.0 M NaCl leukocyte extracts from all patients were considered as antigen solutions. Precipitin tests were performed with use of serial dilutions in saline of antigen solutions and undiluted antibody solutions. All such tests were negative. Similar studies were performed with use of undiluted leukocyte extracts as antigens, and serial saline dilutions of antibody solutions. All such tests were also negative. Equal volumes of test material were used, and tests were observed for 48 hours in the cold before a final reading was made. A representative type of experiment is given in Table III.

The protein content of the leukocyte extract and erythrocyte elution solutions was determined by the biuret method and found to be minute. It was felt that this low level of protein might prevent a visible precipitation reaction from occurring. Accordingly, an attempt was made to demonstrate antigen activity by antibody neutralization, with use of the leukocyte extracts as antigens. Equal volumes of antibody and antigen solutions were mixed and refrigerated at 6° C. for 48 hours. Serial dilutions in saline of the antibody-antigen mixtures and antibody controls were made and tested for agglutinating activity against trypsinized erythrocytes. No loss of antibody activity because of the action of the leukemic leukocyte extracts could be demonstrated by this technique. Rather, hemolysis and/or an increase in titer of trypsinized erythrocyte agglutination was noted in all the sucrose and 2.0 M NaCl leukocyte extract mixtures, and in several of the 0.14 M NaCl extract mixtures. Representative results are summarized in Table IV. It was concluded from these studies that no evidence was demonstrated for antigenic activity in the leukemic leukocyte extracts in relationship to the eluted erythrocyte antibodies found in acquired hemolytic anemia.

The possibility that the antibody coating the erythrocytes in acquired hemolytic anemia is an abnormal protein originating from leukemic leukocytes was then considered. This possibility disregards the autoimmune concept

TABLE II. LEUKOCYTE COUNT, VOLUME OF PACKED CELLS EXTRACTED, AND DIFFERENTIAL COUNT OF EXTRACTED CELLS FROM PATIENTS LISTED IN TABLE I

	PATIENT NUMBER											
	1	2	3	5	6	7	8	9	10	11	12
Leukocyte count	110,000	69,000	78,000	152,000	60,000	190,000	102,000	280,000	224,000	185,000	79,000
Ml. extracted cells	0.8	0.2	0.5	1.25	0.6	1.4	1.1	6.0	1.0	3.1	2.0
Differential count on extracted cells (%):											
Erythroecytes	4	25	5	14	8	6	11	10	12	6	12
Myeloblasts	-	-	-	-	1	-	4	10	-	11	-
Progranulocytes	-	-	-	-	-	-	32	45	1	75	-
Myelocytes	-	-	-	-	-	-	21	24	17	5	-
Metamyelocytes	-	-	-	-	-	-	19	7	20	2	-
Band cells	-	-	-	-	-	1	10	-	36	1	1
Segmented cells	-	-	-	-	-	-	1	4	14	-	3
Lymphoblasts	-	-	-	-	-	-	-	-	-	-	-
Prolymphocytes	-	1	10	5	2	4	-	-	-	-	-
Lymphocytes	96	74	85	81	90	89	2	-	-	-	14
Monoblasts	-	-	-	-	-	-	-	-	-	-	3
Promonocytes	-	-	-	-	-	-	-	-	-	-	20
Monocytes	-	-	-	-	-	-	-	-	-	-	47

Cellular extracts were not made from Patient 4 because of a low leukocyte count.

TABLE III. REPRESENTATIVE RESULTS FROM PRECIPITIN TESTS USING EQUAL VOLUMES OF ELUTED ANTIBODY AND LEUKOCYTE EXTRACT ANTIGEN SOLUTIONS

SALINE DILUTIONS	UNDILUTED SOLUTIONS	TITER*				
		1:1	1:4	1:16	1:64	1:256
Leukocyte Extract Antigen + Antibody						
Patient 1	Patient 1					
0.88 M sucrose	Erythrocyte elution	-	-	-	-	-
0.14 M NaCl	Erythrocyte elution	-	-	-	-	-
2.0 M NaCl	Erythrocyte elution	-	-	-	-	-
Patient 2	Patient 2					
0.88 M sucrose	Erythrocyte elution	-	-	-	-	-
0.14 M NaCl	Erythrocyte elution	-	-	-	-	-
2.0 M NaCl	Erythrocyte elution	-	-	-	-	-
Antibody + Leukocyte Extract Antigen						
Patient 3	Patient 3					
Erythrocyte elution	0.88 M sucrose	-	-	-	-	-
Erythrocyte elution	0.14 M NaCl	-	-	-	-	-
Erythrocyte elution	2.0 M NaCl	-	-	-	-	-
Patient 4	Patient 5					
Erythrocyte elution	0.88 M sucrose	-	-	-	-	-
Erythrocyte elution	0.14 M NaCl	-	-	-	-	-
Erythrocyte elution	2.0 M NaCl	-	-	-	-	-

*All precipitin tests were observed for 48 hours in the cold before a final reading was made.

and would postulate the direct action of a protein affecting erythrocytes, derived from leukemic leukocytes, and acting in a nonspecific manner. An indication that such a mechanism might be present was given in Table IV. In that set of experiments it was found that the addition of leukemic leukocyte extracts to eluted erythrocyte antibodies increased the agglutination titer

TABLE IV. ANTIBODY NEUTRALIZATION STUDIES

ANTIBODY SOLUTION	+ ANTIGEN SOLUTION LEUKOCYTE EXTRACT	TITER TRYPSINIZED ERYTHROCYTES	
		AGGLUTINATION	HEMOLYSIS
Erythrocyte Elution			
Patient 1	Saline control	1:32	-
Patient 1	Patient 1, 0.88 M sucrose	1:64	1:32
Patient 1	Patient 1, 0.14 M NaCl	1:32	1:4
Patient 1	Patient 1, 2.0 M NaCl	1:32	1:32
Patient 3	Saline control	1:8	-
Patient 3	Patient 3, 0.88 M sucrose	1:32	1:32
Patient 3	Patient 3, 0.14 M NaCl	1:8	1:4
Patient 3	Patient 3, 2.0 M NaCl	1:32	1:16
Patient 4	Saline control	1:8	-
Patient 4	Patient 11, 0.88 M sucrose	1:128	1:128
Patient 4	Patient 11, 0.14 M NaCl	1:8	1:4
Patient 4	Patient 11, 2.0 M NaCl	1:128	1:128
Plasma			
Patient 1	Saline control	1:8	-
Patient 1	Patient 5, 0.88 M sucrose	1:32	1:16
Patient 1	Patient 5, 0.14 M NaCl	1:4	-
Patient 1	Patient 5, 2.0 M NaCl	1:32	-

Equal volumes of plasma or erythrocyte eluted antibodies and leukocyte extract antigens were mixed and refrigerated at 6° C. for 48 hours. The agglutination titer was determined with use of serial saline dilutions of these mixtures.

against trypsinized erythrocytes and caused hemolysis. This concept was investigated by testing serial, saline dilutions of the leukemic leukocyte extracts for their ability to agglutinate trypsinized erythrocytes. All leukocyte cell washings were tested and found to be negative except in Patient 1; this positive reaction is believed to be due to plasma contamination. The 0.88 M sucrose extract was found to contain a potent hemolysin and agglutinin in all eleven cases tested. The 2.0 M NaCl extracts contained a potent hemolysin and agglutinin in nine of the eleven cases tested. In five of the eleven 0.14 M NaCl extracts tested, a potent hemolysin and agglutinin were demonstrated. In all cases the hemolysin and agglutinin were active at 37° C., and in most cases it could also be demonstrated at 6° C.; however, a lower titer at 6° C. than at 37° C. was the usual finding. The results of these tests and the highest titer of hemolysis and agglutination activity against trypsinized erythrocytes are given in Table V.

TABLE V. THE AGGLUTINATION AND HEMOLYSIS OF TRYPSINIZED ERYTHROCYTES BY LEUKEMIC LEUKOCYTE EXTRACTS

PATIENT	REACTION	SUCROSE EXTRACT		0.14 M NACL EXTRACT		2.0 M NACL EXTRACT	
		37° C.	6° C.	37° C.	6° C.	37° C.	6° C.
1	Agglutination	1:64	–	–	–	1:32	1:16
	Hemolysis	1:32	1:128	–	–	1:8	1:8
2	Agglutination	1:32	1:8	1:4	1:2	1:8	1:2
	Hemolysis	1:4	–	1:4	–	1:4	1:2
3	Agglutination	1:4	–	–	–	1:4	–
	Hemolysis	1:2	–	–	–	1:2	–
5	Agglutination	1:32	1:8	–	–	1:16	–
	Hemolysis	1:16	1:2	–	–	1:16	–
6	Agglutination	1:32	1:1	–	–	–	–
	Hemolysis	1:4	1:8	–	–	–	–
7	Agglutination	1:16	1:8	1:4	1:2	1:32	1:8
	Hemolysis	1:32	1:8	1:4	–	1:16	1:2
8	Agglutination	1:8	–	–	–	1:1	1:8
	Hemolysis	1:4	1:4	–	–	1:4	–
9	Agglutination	1:128	1:2	–	–	1:64	1:8
	Hemolysis	1:32	1:2	–	–	1:16	1:1
10	Agglutination	1:4	1:4	1:8	1:4	1:2	–
	Hemolysis	1:8	1:2	1:8	1:2	–	–
11	Agglutination	1:64	1:64	1:16	–	1:128	1:64
	Hemolysis	1:128	1:8	1:4	–	1:128	1:2
12	Agglutination	1:16	1:8	1:32	1:4	1:8	1:4
	Hemolysis	1:16	–	1:32	–	1:16	–

DISCUSSION

The assumption that an autoimmune mechanism is responsible for the development of an acquired hemolytic anemia in the course of chronic lymphocytic leukemia is based on the demonstration of an abnormal protein on the surface of the erythrocytes.[5] This protein is thought to be an antibody because of its gamma globulin nature[6] and positive antiglobulin (Coombs) reaction. Although these findings are suggestive, definitive proof of an antibody-antigen relationship is lacking because of the inability to demonstrate an antigen. Stats and Wasserman[7] and Dacie[8] have suggested that the antigen is present in the plasma. Dameshek[9] suggests that the antigen is located in the

erythrocyte stroma where it probably functions as a haptene. However, there is as yet no experimental proof to support these concepts. Lui and Evans[10] have been successful in producing Coombs positive erythrocytes in rabbits by the injection of homologous blood. However, control studies using silica as the injecting substance also resulted in Coombs positive erythrocytes.[11]

A common abnormality seen in the "symptomatic hemolytic anemia" which may complicate many of the lymphomas, plasma cell myeloma, the leukemias, systemic lupus erythematosus, and various tumors is an abnormal cell. It appeared logical to search for an antigen in these abnormal cells. There is some evidence that leukemic leukocytes may act antigenically different than normal leukocytes.[12, 13] In addition, it has been shown that normal leukocytes may carry antibody characteristics not specifically directed toward leukocytes.[14] A search for material antigenically related to antibodies eluted from Coombs positive erythrocytes derived from chronic lymphocytic leukemia patients was made. Leukocytes from various types of leukemia with and without a complicating acquired hemolytic anemia were extracted. Precipitin tests and antibody neutralization studies were performed with use of the eluted antibodies and leukemic leukocyte extracts; all such studies were negative. It is concluded that no antibody-antigen relationship between these two materials was demonstrated utilizing these techniques.

The possibility was considered that leukemic leukocytes may contain material which could directly and nonspecifically react with erythrocytes. This would eliminate the postulated immunologic mechanism and the concept of an autoimmune disease. A nonspecific erythrocyte agglutinating activity of a protein unrelated to erythrocytes has been well demonstrated for the phytohemagglutinin.[15] This type of action would also fit many clinical findings. It is not uncommon to see cases of obvious acquired hemolytic anemia in chronic lymphocytic leukemia characterized by a rapid decrease of erythrocytes, the development of spherocytosis and reticulocytosis, an exponential type of shortened erythrocyte survival time as studied with chromium 51, and negative direct and indirect Coombs tests. It also may be questioned whether the finding of Coombs positive erythrocytes in itself is indicative of an immunologic reaction. A mechanism other than an immunologic one is suggested by the following findings: there is a relative lack of specificity of the coating antibody as determined by its reaction with antihuman globulin sera. Although some degree of specificity has been demonstrated,[16, 17] the degree is in sharp contrast to the specificity encountered in the isoimmune states, where an immunologic mechanism is well documented. Along this line, Crawford and Mollison[18] have demonstrated an immunologic difference between the iso- and autoimmune antibody types. In a more recent study, Komninos and Aksoy[19] injected into rabbits material eluted from Coombs positive erythrocytes derived from one case each of chronic lymphocytic leukemia and lupus erythematosus. Nonspecific antisera were produced which reacted equally well with cells coated with autoantibodies and with cells coated with isoantibodies. It is also questionable whether a positive reaction using antihuman sera directed against normal globulins (Coombs sera) signifies the presence of ab-

normal globulins produced in response to abnormal antigens. Emphasizing our general lack of understanding of the process involved is the study of Coombs and Mourant,[20] who injected various isolated human serum fractions into rabbits to produce antihuman globulin serum. It was found that even the human albumin fraction led to the production in the rabbit of antihuman globulin serum. In addition, there is experimental evidence which casts doubt as to the validity of a positive Coombs test being indicative of an autoimmune process. Muirhead, Groves, and Bryan[21] have utilized phenylhydrazine injections in dogs to produce positive Coombs reactions. The time relationship for the development and loss of the Coombs positive state appeared to rule out an immunologic mechanism.

A search was made for material in leukemic leukocyte extracts which might directly affect erythrocytes. An attempt was made to separate cellular fractions containing cytoplasmic material soluble in 0.88 M sucrose solutions, particulate material with the solubility of ribonucleoprotein with use of 0.14 M NaCl solutions, and material with the solubility of desoxyribonucleoprotein extracted in 2.0 M NaCl solutions. It was found that the 0.88 M sucrose and 2.0 M NaCl extracts, returned to isotonic strength, contained a potent hemolysin and agglutinin when tested against trypsinized erythrocytes. In five cases a similar hemolysin and agglutinin were demonstrated in 0.14 M NaCl extracts. In all positive extracts the hemolysin and agglutinin were demonstrated at 37° C., with variable titers found at 6° C.

The significance of the presence of a hemolysin and agglutinin in leukemic leukocyte extracts is still to be determined. There are several findings which appear to indicate that material eluted from Coombs positive erythrocytes is not similar to the hemolysin and agglutinin found in leukemic leukocyte extracts. The fact that two cases of chronic lymphocytic leukemia, three cases of chronic granulocytic leukemia, one case of acute granulocytic leukemia, and one case of acute monocytic leukemia, all without evidence of an acquired hemolytic anemia, yielded leukocyte extracts with agglutinating and hemolyzing activity suggests a dissimilar nature. The finding of a hemolytic and agglutinating activity in the leukocyte extracts and only an agglutinating action of the eluted antibody further suggests a dissimilar nature. Preliminary data from more detailed studies appear to confirm the impression that different materials are involved.

SUMMARY

1. 0.88 M sucrose, 0.14 M NaCl, and 2.0 M NaCl extracts were made from homogenized leukocytes derived from eleven cases of various types of leukemia. Antibodies were eluted from the red cells of four out of five cases of acquired hemolytic anemia complicating chronic lymphocytic leukemia.

2. With the utilization of precipitin and antibody neutralization techniques, no antigen-antibody relationship could be demonstrated between the leukemic leukocyte extracts and the eluted antibodies.

3. A potent hemolysin and agglutinin against trypsinized erythrocytes were found in all the 0.88 M sucrose extracts, in nine of eleven 2.0 M NaCl extracts, and in five of eleven 0.14 M NaCl extracts.

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URIC ACID BODY POOLS AND TURNOVER RATES IN LEUKEMIA

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PATIENTS with leukemia and polycythemia vera not uncommonly show an increased level of serum uric acid and, at times, present the findings of overt acute arthritis.[1-3] They may also have increased urinary uric acid excretion[4] and, indeed, uric acid calculi and consequent renal failure have been observed.[5] Uric acid, which is the end product of purine metabolism in man, is presumed to increase with an increased rate of destruction of cells.

Recent investigations, using isotopically labeled uric acid, have demonstrated the miscible body pool and rate of turnover of uric acid to be increased in gout.[6, 7] Bishop, Garner, and Talbott[8] determined the body pool and rate of turnover in a series of normal subjects and patients with various diseases. In their one leukemic patient, who was markedly hyperuricemic, an abnormally large miscible body pool and low turnover rate of uric acid were found. In the present study, the body pools, turnover rates, and excretion of uric acid were determined in three leukemic patients by means of uric acid labeled with radiocarbon in an attempt to characterize more clearly the metabolic abnormalities in this disease.

MATERIALS AND METHODS

The subjects of this study, three patients with chronic leukemia, were maintained on a low purine diet for several days prior to and during the experimental period. For at least four weeks before the study, no therapy had been given and all drugs including salicylates had been withheld. On the morning of the first experimental day, 5 ml. of a solution containing 1.69 mg. of uric acid isotopically labeled with radiocarbon having a specific activity of 2 μc per milligram was injected quickly into the tubing of a rapidly running intravenous infusion of 5 per cent dextrose in water. Assuming a uniform distribution of the radioactive uric acid in the total body fluids, an integrated dose of less than 0.002 roentgen equivalent physical whole body irradiation was received by each subject.

Uric acid-2-C^{14} was prepared by the method of Cavalieri, Blair, and Brown.[9] The product was twice precipitated from a lithium hydroxide solution by acetic acid and further purified by ion exchange chromatography with use of a column of Amberlite IRA-411 in the chloride cycle. The uric acid was eluted from the column with 0.01 N hydrochloric acid; its appearance in the eluate was determined by its intense absorption at 290 mμ. The first and last portions of the eluted uric acid were discarded. The remainder was precipitated with glacial acetic acid. The uric acid was prepared for injection just prior to use by dissolving it with an equimolar quantity of lithium hydroxide in pyrogen-free water and passing the solution through a Seitz filter. Blood agar cultures of the solution showed no growth.

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The total urinary output of each patient was collected in twelve-hour periods. A quantitative determination of uric acid by an adaptation of the Brown modification of the Folin method[10] and of creatinine by the Jaffe reaction[11] was carried out on an aliquot of each specimen. Uric acid was prepared for the determination of specific activity by the procedure of Seligson and Shapiro.[12] Crude uric acid was precipitated from about 100 ml. of each urine specimen in the cold after addition of 5 ml. glacial acetic acid. The crystals were dissolved in 10 ml. hot 0.1 N lithium hydroxide solution and diluted to 250 ml. with water. This solution was first passed through a column of Amberlite IRC-120 resin to remove interfering cations. The solution was then chromatographed on a column of Amberlite IRA-411 resin in the chloride cycle, with use of 0.005 N hydrochloric acid as eluent. The column was 1.3 cm. in diameter, 18 cm. high, and contained 15 Gm. of resin. Uric acid, which appeared in high concentration in the 200 to 400 ml. portions of eluate, was precipitated in the cold after addition of glacial acetic acid. A final crystal crop was obtained by acidification with glacial acetic acid of a lithium hydroxide solution of the uric acid crystals. All samples gave a single ultra-violet light-absorbing spot when chromatographed on Whatman No. 1 paper, with use of 40:10:50 butanol-acetic acid-water as developer, corresponding in position to authentic uric acid ($R_f = 0.30$). A thin window (1.3 mg. per square centimeter) GM tube was used for counting, and all counts were corrected to infinite thinness.

The theoretical considerations and assumptions used in the calculation of the miscible pool and turnover rates of uric acid have been discussed in extenso.[6, 8]

PATIENT PROTOCOLS

E. B., a 42-year-old man, 144 pounds in weight, was found to have chronic lymphatic leukemia on bone marrow biopsy 4 years previously. He had been treated with nitrogen mustards and radiation in the past. Prior to the last course of radiation therapy the white cell count was 11,300 with 59 per cent lymphocytes. During this study, the white cell count was 2,400, the basal metabolic rate +19 and +30, and the serum uric acid 9.5 mg. per cent. There were no symptoms or signs of gout. Blood urea nitrogen was 15 mg. per cent.

A. B., a 58-year-old man, 117 pounds in weight, was found to have chronic lymphatic leukemia by bone marrow biopsy one year previously. Prior treatment included courses of ACTH and TEM (triethylenemelamine); the last course of TEM was concluded one month prior to this study. The white count was 5,000 with 51 per cent lymphocytes, basal metabolic rate +9 and +17, and the serum uric acid was 5.7 mg. per cent. There were no symptoms or signs of gout.

C. D., a 57-year-old man, 140 pounds in weight, was found to have chronic myelogenous leukemia by bone marrow biopsy one year previously. Prior treatment included a course of urethane. This patient was a well-controlled diabetic. The white count was 45,000, the protein-bound iodine 5.8 mcg. per cent, and the I^{131} uptake 36 per cent in 24 hours. The serum uric acid was 8.0 mg. per cent and the blood urea nitrogen was 21 mg. per cent. There were no symptoms or signs of gout.

RESULTS

The results in our patients are given in the accompanying table (Table I). The specific activity regression curves from which these values were calculated are plotted in Fig. 1. The turnover rate, in pools per day, is obtained from the slope of the regression curve. The intercept of the curve with the ordinate is the natural logarithm of the specific activity of uric acid in the body immediately after injection of the isotopically labeled uric acid. The miscible body pool of uric acid is calculated from this value and the known amount of isotope administered. The total daily turnover is the product of

TABLE I. URIC ACID: BODY POOLS AND TURNOVER RATES, CONCENTRATION IN BODY WATER AND SERUM, URINARY EXCRETION, AND RECOVERY OF ADMINISTERED ISOTOPE

PATIENT	WT. (KG.)	SERUM CONCENTRATION (MG. PER CENT)	MEAN CONCENTRATION IN BODY WATER (MG. PER CENT)	MISCIBLE BODY POOL (MG.)	RATE OF TURNOVER (POOLS PER DAY)	TOTAL DAILY TURNOVER (MG.)	AVERAGE URINARY EXCRETION (MG. PER DAY)	RECOVERY OF LABELED URIC ACID IN URINE (PER CENT)
Normal*		3.0 to 6.0	1.4 to 2.8	700 to 1,400	0.5 to 0.9	350 to 1,300	300 to 500	60 to 100
A. B.	53	5.7	2.1	748	0.908	679	512	74
C. D.	64	8.0	3.2	1,360	0.636	865	629	63
E. B.	65	9.5	6.0	2,380	0.996	2,370	1,830	77
W. de R.†		20		5,489	0.398	2,190		
Gouty‡		6.0 to 20	4.0 to 35	2,000 to 18,000	0.35 to 0.51	700 to 9,000	300 to 1,000	34 to 75

*Normal values were taken from the literature.[6-8]

†Patient reported by Bishop, Garner, and Talbott.[8]

‡The ranges for gout were taken from the literature and include extremely high values reported in some patients with tophaceous gout.[6]

the miscible body pool and the turnover rate. The mean concentration of uric acid in body water was calculated from the body pool after assuming that the total body water was equal to 70 per cent of the body weight.

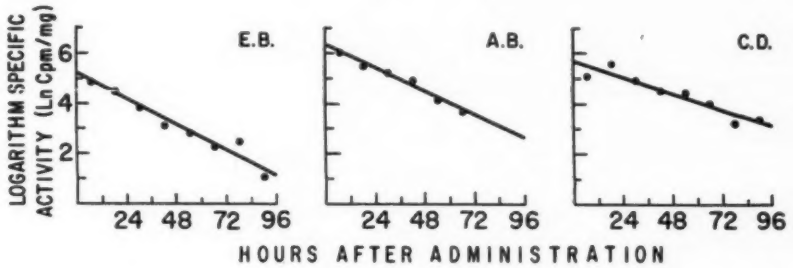

Fig. 1.—The specific activity regression curves of labeled urinary uric acid following injection of a tracer dose. Miscible body pool is calculated from the intercept and rate of turnover from the slope.

DISCUSSION

Using values reported in control subjects[6, 8] as normal, we found one of our patients to have a miscible body pool of uric acid in the normal range; a second patient had a pool size at the upper limit of normal; and the pool size was abnormally large in the third. The size of the miscible pool roughly paralleled the level of serum uric acid.

The mean concentration of uric acid in the body water of two of our patients was in the range of normal subjects reported previously.[6] One of our patients, E. B., had a somewhat elevated concentration in body water, but this was still less than his serum uric acid. A high concentration of uric acid in body water, particularly if it is greater than the serum uric acid, is evidence for the accumulation of uric acid within cells or in insoluble but miscible depots, a finding in cases of tophaceous gout.

The rate of turnover of uric acid was in the normal range in all of our cases; it was in excess of the upper limit of 0.510 pools per day observed in gouty patients.[8] The recovered labeled uric acid from the urine falls in the range reported for normal subjects. A portion of the endogenous uric acid appears to be eliminated by the gastrointestinal tract or to be metabolized by the body or by the intestinal flora. In patients with gout, a larger amount of uric acid is diverted from the urine and recoveries as low as 34 per cent have been reported.[7]

In the subject with chronic myelogenous leukemia studied by Bishop, Garner, and Talbott,[8] the miscible body pool was found to be 5,489 mg. and the turnover rate 0.398 pools per day. The serum uric acid was reported to be as high as 20 mg. per cent. These values, in contrast to those found in our own patients, are similar to the values found in patients with gout. It should be noted that the determinations in this patient were made with uric acid labeled with N^{15} rather than with C^{14}-labeled uric acid used in the present cases.

Our leukemic patients appear to be able to excrete the larger amounts of uric acid which are formed, thus keeping the body pool down and the turnover rate up. W. de R., the patient of Bishop and associates,[8] while producing almost the same amount of uric acid as E. B., had a higher pool and therefore a lower turnover rate. Bishop's patient may have sustained renal damage which would explain the higher pool. It may be that the leukemic patient is able to keep his body pool of uric acid under reasonable control until he sustains renal damage, whereupon the pool size becomes very large and even gout may develop.

SUMMARY

The miscible body pool and rate of turnover of uric acid were measured in three patients with leukemia with use of uric acid labeled with radiocarbon. The pool sizes were normal, borderline, and elevated, respectively, in the three patients and related to the serum uric acid concentrations. The mean concentration of uric acid in body water was elevated in one patient. The turnover rates and the per cent recovery of labeled uric acid in the urine were normal. The findings in these cases were contrasted with those of a leukemic patient reported by Bishop and associates and with those reported for patients with gout.

The encouragement and support of Dr. J. Russell Elkinton and Dr. Ralph Jones, Jr., are gratefully acknowledged.

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LABORATORY METHODS

THE USE OF CAPILLARY BLOOD FOR ESTIMATING RENAL CLEARANCE OF INULIN AND GLUCOSE EXCRETION BY MEANS OF AN ANTHRONE PROCEDURE

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IN A previous publication[1] a method was described to estimate microgram quantities of inulin by means of the anthrone reagent. In the present study, the procedure has been extended so that glucose can be determined simultaneously and the combined method be used for blood and urine analysis. This has made it possible to estimate both inulin and glucose in a 0.2 ml. sample of blood when the concentration of these carbohydrates are at levels usually employed for renal function studies.

The method presents many advantages in clinical experimentation. It allows the determination of inulin clearances in the presence of changing blood sugar values without the need for "yeasting." In addition, it is sufficiently sensitive to permit the use of capillary blood for analysis. Since the blood sugar values in capillary blood approximate those in the artery, errors caused by the use of venous blood can be avoided.[2]

Practical application of the procedure for physiologic experimentation introduced several problems related to the use of capillary blood. These included, first, the question whether whole blood, so conveniently obtained by puncture of a fingertip, could be used instead of plasma which is usually measured in clearance studies. Second, it was necessary to establish whether inulin in capillary blood actually came into equilibrium with the inulin in arterial blood during the "constant infusion technique" as ordinarily employed.[3] Finally, it was necessary to determine the limitations of the method when actually applied to the study of renal function. A solution of these problems forms the basis for this work. Several clinical experiments performed to determine the effect of Priscoline on the glomerular filtration rate are reported.

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METHODS

Chemical Procedures.—Precipitation of the proteins was accomplished by the introduction of a 0.2 ml. sample of whole blood into 4.8 ml. of freshly prepared dilute tungstic acid solution as used by Peters and Van Slyke.[4] For plasma, 0.2 ml. was introduced into 3.8 ml. of the tungstic acid solution in centrifuge tubes. These were stoppered with rubber stoppers free of starch and shaken *vigorously* for about one minute. The precipitate was allowed to settle and then centrifuged. For the anthrone procedure, 1 ml. of the filtrate was introduced into Coleman spectrophotometer tubes and 1 ml. of water was added. The tubes were treated as previously described[1] by chilling in an ice water bath, by the slow introduction of 1 ml. of chilled 95 per cent sulfuric acid, and after stirring with a glass rod, by the further careful addition of 3 ml. of chilled anthrone reagent (0.4 per cent).* The mixtures were thoroughly stirred and after being allowed to come to room temperature, the tubes were placed into a water bath at 56° C. for exactly 10 minutes. The reaction was stopped by rapid immersion in a tap water bath and readings were made in the Coleman spectrophotometer at 620 mμ. The color at this point was predominantly due to inulin. After further stirring with glass rods, the tubes were placed in a boiling water bath for exactly 12 minutes. The reaction was stopped in a tap water bath, and the tubes were again read in the Coleman spectrophotometer but this time at 540 mμ. The last reading represented the glucose content plus inulin. A 2 ml. water blank, a glucose standard in which 2 ml. contained 100 mcg., and an inulin standard in which 2 ml. contained 20 mcg. were all treated as above. Although not necessary, another 2 ml. standard containing a combination of 20 mcg. of inulin and 100 mcg. of glucose was included as a check on the method. When properly performed, the optical densities of this combined standard obtained after being heated at 56° C. and 100° C. will equal the sums of the readings of the two separate standards at 56° C. and 100° C., respectively.

The relative inulin and glucose contents in the filtrates can be calculated by substituting and solving the following simultaneous equations for X and Y. This equation was derived after finding that standard curves of optical density versus concentration pass through the origin when plotted for each component.

$$OD_{C56^\circ} = \frac{OD_{I56^\circ} \cdot X}{20 \text{ mcg.}} + \frac{OD_{G56^\circ} \cdot Y}{100 \text{ mcg.}}$$

$$OD_{C100^\circ} = \frac{OD_{I100^\circ} : X}{20 \text{ mcg.}} + \frac{OD_{G100^\circ} \cdot Y}{100 \text{ mcg.}}$$

where OD_{I56° or OD_{I100° = optical density observed for the 20 mcg. inulin standard after being in water bath at 56° C. and 100° C., respectively

OD_{G56° or OD_{G100° = optical density observed for the 100 mcg. glucose standard after being in water bath at 56° C. and 100° C., respectively

OD_{C56° or OD_{C100° = optical densities observed for the combined inulin and glucose standard or for the optical densities of the filtrates after being in the water at 56° C. and 100° C., respectively

X = micrograms of inulin, and Y = micrograms of glucose.

Collection of Capillary Blood Specimens.—Blood for analysis was obtained from warmed fingertips stuck with a Bard Parker No. 11 blade so that the blood flowed freely. For whole blood analyses, commercially available 0.2 ml. pipettes were employed to collect the blood. For capillary plasma, glass tubing of 4 mm. bore was cut into 6-inch lengths and an open point made at one end. A 2 per cent potassium oxalate solution was drawn into these tubes until they were one-quarter full and then they were placed into an oven at 100° C. until dry. Super heating must be avoided. When properly dried, the oxalate will be found distributed as a powder throughout the tube. Employing these oxalated tubes as pipettes, approximately 0.5 ml. of blood was obtained and each end was completely sealed with Peyseal.† The speci-

*This reagent was prepared fresh daily by dissolving 0.4 Gm. of anthrone in 100 ml. of 95 per cent sulfuric acid. It was allowed to stand at least 3 hours before using.

†Peyseal (Fischer Scientific Company).

mens were then centrifuged until there was a separation of the plasma from the cells. At the line of separation the glass was cut and the section containing plasma was emptied into micropipettes calibrated to contain 0.2 ml. of plasma.

Clearance Technique With Inulin.—Details concerning the infusions will be given in the discussion of results. For the collection of urine, soft rubber catheters with five holes were employed. The bladder was washed with 30 ml. of saline to complete each period of urine collection. With the dosages used, a 1 to 200 or 250 dilution of urine was usually satisfactory for analysis.

RESULTS

The anthrone procedure described above can be considered in two sections. The first part of the method predominantly determined inulin and no difficulty was found in estimating inulin in concentrations up to 45 mg. per cent. The comparatively small color developed by glucose at 56° C. was not only constant but also additive to that produced by inulin. Thus, when the glucose concentration of plasma was constant, determination of inulin could be made directly (without using the formula) by subtracting a "plasma chromogen blank" in the manner employed in other chemical analyses determining inulin.[5] In Table I are the results obtained by this abbreviated anthrone method as well as values obtained by the Schreiner method[5]; the comparative results are well within limits allowable for experimental error.

TABLE I. RECOVERY OF INULIN IN FILTRATES BY THE ANTHRONE MICRO METHOD

		INULIN CONTENT (MG. %)		
	FILTRATES PREPARED FROM	AMOUNT ADDED	DETERMINED BY ANTHRONE METHOD	RECOVERY (%)
Recovery of inulin added to filtrates in vitro	W.B.	9.4	9.8	104
	W.B.	15.7	15.4	98
	W.B.	18.8	19.3	103
	Plasma	19.8	20.1	101
	Plasma	21.6	21.8	101
	Plasma	39.6	40.2	101
		DETERMINED BY SCHREINER METHOD	DETERMINED BY ANTHRONE METHOD	
Comparison with results obtained by Schreiner method	Plasma	27.9	28.6	102
	Plasma	30.1	30.5	101
	Plasma	34.7	34.9	101
	Plasma	40.9	41.3	101

"Chromogenic blank" employed to calculate results.

The second portion of the procedure which estimates the combined value of glucose and inulin was found to have many limitations. This was due to the nature of the chemical reaction, because during the boiling, color formation arising from the reaction of glucose with anthrone reagent proceeded from a negligible amount to a maximum plateau level.[6] Simultaneously, during the boiling, there was degradation of the color due to inulin which was already maximal after the first procedure. Thus, it was found that after 12 minutes in the boiling water bath, inulin produced an optical density that was higher than an equal quantity of glucose.* When mixed, the optical densities were found

*A new anthrone procedure for the estimation of levulose and glucose has been developed by Sass and associates which eliminates this difficulty, thus avoiding the need for a formula. Several experiments in this work have already been performed with the new procedure.

to be additive but only in a range limited by the total amount of the carbohydrate present. Combinations up to 20 mcg. inulin and 100 mcg. glucose mixed in a single standard yielded an optical density at 540 mμ that equaled the sum of the optical densities of each determined individually. Up to this concentration and slightly higher the relative content of inulin and glucose was accurately calculated by employing the equations described in "Methods."

The above optimal concentration is found in filtrates prepared by a 1 to 20 dilution of plasma containing 40 mg. per cent inulin and 200 mg. per cent glucose. This plasma level is adequate for studies of inulin clearance, but glucose concentration at least two to three times higher is required to determine the maximum tubular reabsorption of glucose (TMG). With such elevated glucose concentrations, an extension of the method was found necessary. When optical densities obtained after the step in the boiling water bath were above optimal range, dilutions could be made with a specially prepared anthrone solution to bring the optical densities to the proper level. Such dilutions were found to yield accurate proportional decreases; for instance, the addition of an equal quantity of the anthrone diluent to a preparation decreased the optical density exactly to half. Thus, the formula could be still used to calculate the relative inulin and glucose content as long as $OD_{C100°}$ was corrected for the dilution.

The anthrone diluent was similar to the water blank and could be prepared at any time by the slow addition of the anthrone reagent to distilled water in a tap water environment with use of proportion of four volumes of the former to two volumes of the latter. This mixture was thoroughly mixed and then heated in a boiling water bath for 10 to 12 minutes. After cooling, the addition of this reagent for diluting purposes created no heat since the sulfuric acid concentration was the same as in the preparations that were diluted.

DISCUSSION

The anthrone method was found to be highly sensitive and accurate in determining both glucose and inulin in the same solution after the difficulties mentioned above were cleared up. The application of the procedure to clinical experimentation also introduced many problems that needed additional studies. These will be discussed individually.

Capillary Plasma Versus Whole Blood for Estimating Inulin.—Results in clearance studies are usually expressed in terms of the amount of plasma cleared. When using capillary blood for such studies, if one could employ whole blood obtained directly from a finger puncture, it would greatly simplify matters. Since little or no inulin is supposed to enter the erythrocytes, it seemed possible to calculate plasma inulin concentrations by hematocrit corrections of whole blood values. This possibility was first explored by experiments performed in vitro. Plasma was separated from 10 ml. samples of venous blood. To this plasma, a known amount of inulin in 0.2 ml. saline was added and well mixed. After 0.2 ml. of the mixture was removed for analysis, the blood was reconstituted by the addition of the cellular fraction, again well mixed, and

then centrifuged. A second 0.2 ml. sample of plasma was withdrawn for analysis. The results of representative experiments are given in Table II, *A*; from 8 to 10 per cent inulin was removed during the procedure.

TABLE II. INULIN CONTENT OF PLASMA—INFLUENCE OF BLOOD CELLS

A. In vitro experiment. Effect of adding R.B.C to plasma containing inulin		PLASMA INULIN BEFORE ADDING R.B.C. (MG. %)	PLASMA INULIN AFTER ADDING R.B.C. (MG. %)	% REMOVED
		17.4	15.6	−10.3
		21.1	19.4	− 8.1
		29.0	26.2	− 9.7
		40.2	36.3	− 9.7
B. In vivo experiment. Blood obtained from 3 patients receiving infusions of inulin	INULIN CONTENT DETERMINED IN		PLASMA INULIN CALCULATED BY HEMATOCRIT FROM WHOLE BLOOD (MG. %)	% DIFFERENCE (CALCULATED MINUS DETERMINED OVER DETERMINED)
	WHOLE BLOOD (MG. %)	PLASMA (MG. %)		
	8.9	17.0	18.0	+ 5.9
	8.6	16.3	17.9	+ 9.8
	8.9	16.2	17.8	+ 9.9
	13.0	20.9	23.6	+12.9
	12.9	19.5	23.0	+17.9
	12.9	19.9	22.6	+15.5
	16.8	26.4	29.5	+11.7
	14.0	23.5	25.9	+10.3

This percentage of inulin "trapped" with the erythrocyte seemed rather constant and the slight variations in results might well have been due to mechanical faults in mixing during conduction of the experiments. Thus the problem was further explored in vivo with blood collected from patients receiving infusions of inulin. Filtrates were prepared from both the whole blood as well as the plasma fraction of the same specimen of venous blood of such patients. The venous hematocrit of each specimen was also determined. In Table II, *B* are given the results of the concentrations of inulin directly determined in plasma filtrates as compared with plasma values calculated by means of hematocrit corrections of the whole blood analyses. As expected, the calculated plasma concentration of inulin exceeded that directly estimated. The results in some experiments suggested that by allowing a correction of 9 to 10 per cent for trapping of inulin by the erythrocytes, an adjustment of whole blood values by hematocrit correction might be employed. However, as shown in Table II, *B*, unexplained additional differences amounting to 4 or 5 per cent were encountered. To avoid such an increase in error, it was concluded that plasma best be used for inulin estimation despite the additional work involved in separating capillary blood as described in the method.

Equilibration of Inulin in Capillary Plasma During the "Constant Infusion Technique."—It is generally agreed that best results are obtained when concentration of inulin is kept at an unchanging level during the performance of clearance tests.[7, 8] For this purpose, a "priming dose" of inulin is given, calculated to effect a desired concentration of inulin throughout the extra-

cellular space. This is followed with a "sustaining" intravenous infusion given at a constant rate, delivering a sufficient amount of inulin per minute to replace that excreted in the urine. For clinical work, Goldring and Chasis[3] found it was possible to eliminate individual calculations of required inulin; these authors recommended standard amounts used for all patients. This was done in our clinical experiments. The "priming" dose consisted of 30 ml. of

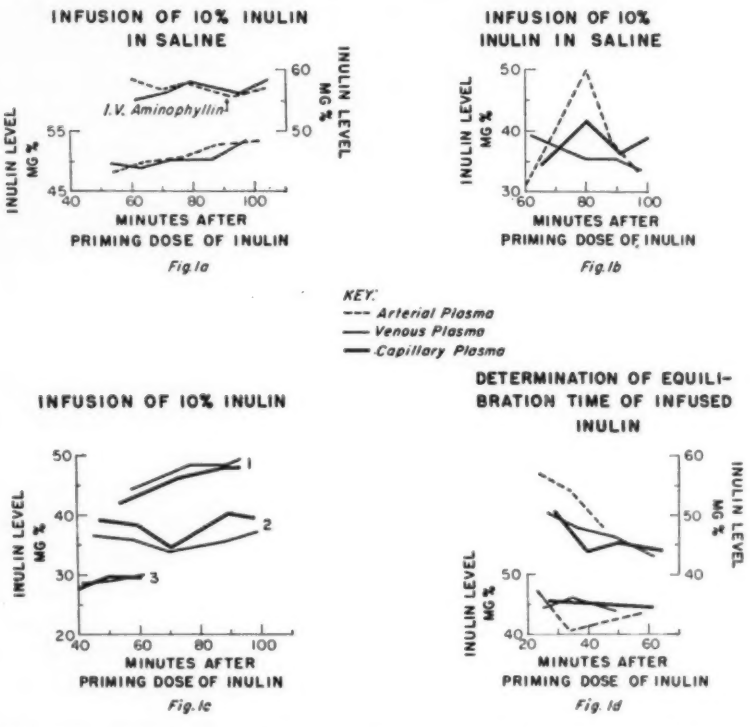

Fig. 1.—Plasma inulin concentrations during sustaining infusion of 10 per cent inulin in 500 ml. saline at various intervals after priming dose of 30 ml. of 10 per cent inulin. Rate of infusion = 6 ml. or 6.9 ml. a minute.

a, Equilibrium of the inulin concentration in arterial and venous plasma of two patients during an infusion.

b, Lack of equilibrium due to restlessness of a patient while receiving an infusion through a Graubard flowmeter. The inulin content found in venous plasma gave no indication of the irregularity found in arterial and capillary plasma.

c, Equilibrium of the inulin concentration in capillary and venous plasma when the infusion flow was constant. In Patients 1 and 3, the Graubard flowmeter; in Patient 2, the infusion pump.

d, Lack of equilibrium of inulin concentration in arterial, capillary, and venous plasm of two patients prior to 50 minutes of infusion.

10 per cent inulin and the "sustaining infusion" contained the proportionality 70 ml. of 10 per cent inulin in 500 ml. of 10 per cent glucose given at 6.0 ml. or 6.9 ml. per minute. Plasma inulin levels between 35 mg. and 55 mg. per cent, depending on the size of the patient, were attained by these doses and were optimum for the anthrone procedure.

The efficiency of the infusion technique was checked by determining the plasma inulin content of blood samples obtained simultaneously from indwelling Riley needles in the brachial artery and median cephalic vein of the same arm. In Fig. 1*a* are the data obtained during the course of two experiments

in which the flow of the sustaining infusions of inulin was regulated by a constant infusion pump* at a constant rate of 6.9 ml. a minute. It can be seen that after equilibration was attained, the arterial level of inulin remained constant. Furthermore, the concentration of inulin in the peripheral venous plasma approximated that in arterial plasma. Since it is the arterial plasma which is actually cleared by the kidney, such an equilibrium is required if venous blood is used for analysis.

The constancy of the "sustaining" infusion was found to be of utmost importance to maintain this equilibrium. This has been emphasized by Brun and associates,[9] who studied arteriovenous differences. With the infusion pump, the flow was always maintained at a steady rate. This was not always found to be true with use of a Graubard-Peterson† flowmeter in which the rate of flow depends on the gravity of the solution slowed down by impedance in the apparatus. Although constant rates were usually obtained with this apparatus when an intravenous needle of 18 gauge or wider was used, restlessness on the part of the patient was observed to change the inflow rate as much as 2 or 3 ml. per minute for short periods. The results obtained in such a case is shown in Fig. 1*b* where restlessness of the patient was reflected by marked changes in the inulin concentration in arterial plasma. The venous plasma estimated simultaneously did not reflect the arterial change; in fact, a false impression of constancy was obtained from the "smooth" appearance of the venous curve. Under such conditions, the inulin content in the capillary plasma varied like that of the arterial plasma. In clearance studies, capillary blood specimens were taken at the midpoint of each period; the constancy of inulin values was a guarantee of the constancy of the infusion.

When the rate of inflow of the "sustaining" solution was steady, a close correspondence between the inulin content of the capillary plasma and venous plasma was obtained whether the flowmeter or the pump was employed. Three representative experiments are shown in Fig. 1*c*. In one, capillary blood was slightly higher and in another, the venous, but the difference between the two was rarely as great as 5 per cent and usually less than 3 per cent. Intravenous administration of aminophylline (0.5 Gm.) and Priscoline (50 mg.) did not increase the capillary-venous relationship.

The studies represented in Fig. 1*d* were designed to determine how long a time after the priming dose was necessary to insure a proper equilibrium. It can be noted that the venous, arterial, and capillary curves approach each other by fifty minutes after which the equilibrium was maintained. As noted above, the venous curves remained "steady" in the face of variable arterial and capillary concentrations and gave no clue that equilibrium was not attained.

The Use of Glucose Solutions as a Vehicle for Inulin Infusion.—The anthrone method, by simplifying the estimation of inulin in the presence of glucose, allows the use of glucose solutions rather than saline for the infusions. This introduces another problem.

*Process and Instrument Company, Brooklyn, N. Y.
†Fischer and Porter, Hatboro, Pa.

TABLE III. INULIN CLEARANCES AND URINARY FLOW BEFORE AND AFTER INTRAVENOUS PRISCOLINE (50 MG.)

PERIOD		PATIENT 1-R.M.		PATIENT 2-M.S.		PATIENT 3-M.L.		PATIENT 4-H.A.		PATIENT 5-L.L.		PATIENT 6-J.M.	
		C_{IN} ML./MIN.	U ML./MIN.	C_{IN} ML./MIN.	U ML./MIN.	C_{IN} ML./MIN.	U ML./MIN.	C_{IN} ML./MIN.	U ML./MIN.	C_{IN} ML./MIN.	U ML./MIN.	C_{IN} ML./MIN.	U ML./MIN.
Before	I	115	5.8	91	10.4	120	6.3	140	1.1	99	1.1	54	9.6
Priscoline	II	116	3.8	97	10.0	121	7.8	132	1.8	83	0.3	43	6.7
After	I	118	4.0	97	8.0	116	5.0	161	2.3	124	3.2	30	3.5
Priscoline	II	111	6.6	88	8.1	118	5.2	158	7.5	137	4.2	33	4.1

C_{IN} represents inulin clearance; U = urine flow.

When 10 per cent glucose is given intravenously at 10 ml. a minute to prehydrated patients, a "diuresis curve" is obtained, the increase in urine flow usually commencing in 30 minutes and reaching a crest in 85 ± 32 minutes.[10] In the present study the rate of flow of the infusion was kept at 6 ml. a minute with the flowmeter or at 6.9 ml. a minute with the infusion pump to avoid excessive diuresis. At this rate, a peak of urine flow was reached at the same time. After allowing a waiting time of 50 minutes for equilibration of the inulin, the first two clearance periods of 10 minutes length were occasionally performed before or at the time of the upswing of a diuresis curve. As pointed out by Smith,[7] a rapid change in the flow of urine is often accompanied by unexplained increases in the glomerular filtration rate. That this can lead to erroneous results is demonstrated in Table III reporting studies in which 50 mg. of Priscoline was given by slow intravenous injection to several patients after two control clearance periods. In the first three patients who were well hydrated and had adequate urine flows at the onset, no changes in the glomerular filtration rate were observed following the administration of the drug. However, in Patients 4 and 5 with initially low urinary flows, the Priscoline seemingly increased the inulin clearance rate, although this effect was probably due to the coincidental onset of a water diuresis due to the infusion. The reverse was found in patient 6 who was coincidentally studied on the decline of a diuresis curve probably caused by apprehension. Thus it was found necessary to plan for the proper prehydration of a patient in relation to the experimental period to obtain a urine flow of at least 2 ml. per minute when the first clearance periods of short interval were made.

Blood Glucose Values for Tmg.—When the 10 per cent glucose sustaining infusion was given at 6 ml. or 6.9 ml. a minute, the capillary plasma glucose concentration in normal individuals rose to 190 mg. to 220 mg. per cent by one hour. Continued infusion raised the plasma glucose only slightly. This fell short of the amount required to saturate the tubules and no glycosuria was generally found. In diabetics the plasma sugar rose much higher (300 to 450 mg. per cent) depending on the severity of the diabetes; marked glycosuria was found in all. Thus, in normal individuals and in milder diabetics additional booster injections of 50 per cent glucose were needed previous to the onset of the clearance periods during which the TMG was determined.

In a previous study,[11] it was found that fasting blood sugar values as determined by anthrone were higher than "true glucose" values obtained by the Somogyi-Nelson method. This difference varied from patient to patient and amounted to as much as 26 mg. per cent in some cases. During the constant glucose infusions, however, this difference was constant for each patient at various elevated blood sugar levels. Thus, to obtain "true blood glucose" values, one or two filtrates in each experiment were also analyzed by the Somogyi-Nelson procedure. The difference when found (usually 15 to 30 mg. per cent) was subtracted from all the anthrone plasma values to obtain a corrected "true plasma glucose."

CONCLUSIONS

The anthrone reagent can be employed to determine both inulin and glucose in microgram quantities when present in the same sample. The method makes it possible to estimate these carbohydrates in capillary plasma obtained by finger puncture during renal function studies. After "priming" with inulin, the inulin content of capillary plasma comes into equilibrium with that of the artery and vein within 50 minutes in most cases. This equilibrium is maintained if the infusion is kept at a constant rate. The anthrone method, by estimating glucose simultaneously, allows the use of glucose solution as a vehicle for the inulin. The intravenous use of glucose solutions, however, can lead to quick swings in the rate of urine flow and can lead to variation in the rates of inulin clearance unless provisions are made to have an adequate urine flow throughout the test. At all blood sugar levels, the anthrone values are usually higher than "true blood glucose"; since this difference is constant, a correction can be easily made.

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THE PRESERVATION OF BLOOD FOR THE PURPOSE OF TRANSFUSION

II. The Effect of the Rate of Cooling on the Preservation of Erythrocytes

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INTRODUCTION

OF THE various steps in the technique of collection and storage of blood for transfusion, the rate of cooling of the blood is one more often left to chance. The prevailing technique in most blood banks is to collect the blood in a cooled bottle, containing the anticoagulant, to place the bottle in a refrigerator with an air temperature varying from 4 to 7° C., and to allow the blood to cool.

A more rapid and reproducible rate of cooling than obtained with this and similar procedures has been suggested to obtain a better control of enzymatic activities, which are generally considered to be a main factor in the preservation of blood. It was soon noted that when the rate of cooling was very rapid, posttransfusion survival of the red cells was poor. The studies here reported were instituted to determine more exactly the effect of the rate of cooling on red cell preservation.

TECHNIQUE

The blood for these studies was collected in plastic bags and in the conventional glass bottles, containing as anticoagulant ACD solution. The technical procedures for the in vitro studies are those described in a previous paper.[1] The technique for tagging of red cells with radioactive sodium chromate likewise has been published previously.[2]

In our experience it is very difficult to obtain by gravity a uniform rate of blood flow into either plastic containers or glass bottles. When blood from venipuncture is collected in plastic containers by gravity, the flow is slower than that obtained with the glass bottles.

The measure of the amount of blood being collected in plastic containers is generally obtained by weighing. This is not feasible when the plastic container must be maintained in continuous agitation in a constant temperature bath, to insure a reproducible procedure. In addition some of the plastic containers become so rigid at refrigerator temperature that collection of the full amount of blood at such temperature is very difficult.

To control the procedure of collection of blood we devised an apparatus for the purpose of (1) drawing blood at a controlled uniform speed, (2) cooling blood at any

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desired rate, (3) mixing blood with anticoagulant by gentle, uniform motion, (4) maintaining blood at any desired temperature during collection, and (5) measuring exactly the amount of blood drawn into plastic containers.

The apparatus consists essentially of a circulating water bath, which can be maintained at any temperature between 1 and 37° C. (Fig. 1). This water tank accommodates a light metal spool with fins, around which is coiled the plastic tubing (*A*) through which the blood is collected. The length of this tubing can be varied at will. A water-tight plastic cylinder (*B*) contains the plastic collection bag (*C*) on a rigid support. One end of this cylinder is rocked through a 15° arc during collection and the rate is approximately 12 full excursions per minute. The cylinder is provided with a rubber diaphragm which fits tightly around the neck of the bag. Through rubber tubes the cylinder is connected with the circulating water tank and with a graduated cylinder (*D*) which is connected with a vacuum pump. A small vacuum pump, a circulating water pump, and a mechanical device for rocking are contained beneath the circulating cold water tank, along with a waste water receptacle.

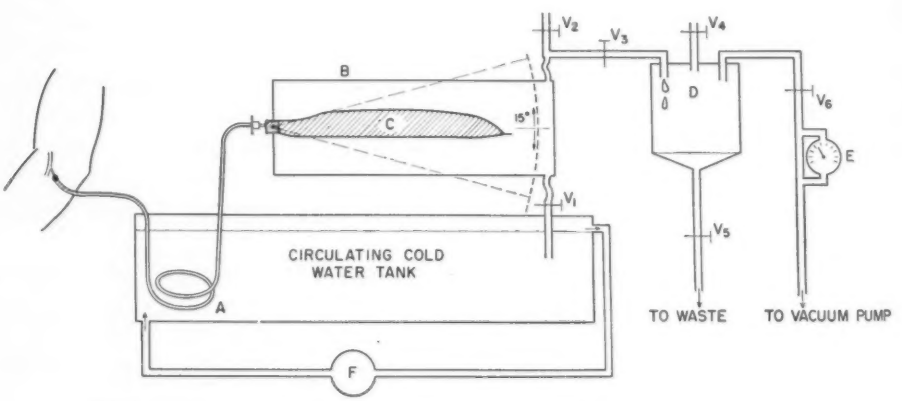

Fig. 1.—Apparatus for collection and cooling of blood at constant rate.

During operation the bulk of the coil of plastic tube can be maintained at room temperature or is immersed in the water of the tank at any desired temperature. When more rapid cooling is desired, the plastic coil may be replaced with one made of metal.

By proper manipulation of Valve V-3, an even, adequate flow of blood can be maintained. An immediate evaluation of the rate of bleeding can be obtained by observing the dripping of water and the rising of its level in the graduated cylinder (*D*). When glass containers are used, they are connected through an air filter directly to the source of controlled vacuum and maintained in gentle agitation while immersed in the water bath at the desired temperature.

Three rates of cooling were used in collecting the blood for the experimental study. They are designated as: slow, moderately rapid, and very rapid.

Slow Cooling.—Slow cooling was designed to reproduce as much as possible the conditions generally obtained in blood bank practice. For this purpose blood was collected at room temperature in a glass bottle containing the ACD solution, previously cooled in the refrigerator. Under these conditions the temperature of the citrated blood at the end of the collection period is generally around 30° C. ± 2°. The bottle is then placed with six other similar bottles in an air-cooled refrigerator, where at least 36 other bottles of blood are already stored and cooled. With moderate air circulation the cooling of blood proceeds as follows: 3 hours: 15° C.; 6 hours: 11° C.; 8 hours: 9° C. It requires generally between 12 and 15 hours for the temperature of the blood to reach that of the refrigerator, maintained at 5 to 7° C.

Moderately Rapid Cooling.—This rate is obtained with the use of a coil of plastic tubing, approximately 4 mm. outside diameter and 225 to 260 cm. in length. This coil is im-

mersed in a circulating water bath maintained at 0.7° C. ± 0.3° C. Under these conditions and with a rate of blood flow of 100 ml. per minute the blood is cooled to a temperature of 8° to 14° C. at the end of collection. The glass or plastic bag is then immersed in chipped ice, where a temperature of 5° C. is obtained in about one-half hour.

Very Rapid Cooling.—This rate is obtained with the use of a stainless steel coil, 150 cm. long with an o.d. of 3.9 mm. and a wall thickness of about 0.3 mm. This coil is connected by means of plastic tubing to metal needles at both ends. When this coil is immersed in a water bath cooled to 0.7° C. as stated above and with the same rate of flow, the blood is delivered to the container at 2° C. ± 1 in less than 4 seconds.

RESULTS

In Vitro Studies.—The in vitro tests which reflect the red cell glycolytic activity, such as the rate of dextrose disappearance, formation of lactic acid, and concentration of adenosine-triphosphate, do not reveal as a rule essential differences between blood collected from the same donor but with various rates of cooling. The same is true of blood collected from various normal donors varying the rate of cooling. Differences, however, are often revealed by tests measuring the integrity of the cell membrane. Thus, the supernatant plasma hemoglobin may be higher and the osmotic fragility increased in the blood very rapidly cooled as compared with blood collected with a moderately rapid or slow rate (Table I).

TABLE I

	SLOW COOLING		VERY RAPID COOLING	
	FRESH	STORED 21 DAYS	FRESH	STORED 21 DAYS
Plasma Hgb. (mg./100 ml.)	8	136	15	137
% Hemolysis in 0.63% NaCl	1	6	2	4

The specimens of blood used in the experiment reported above are the same used in the in vivo studies shown in Fig. 2.

	SLOW COOLING STORED 21 DAYS	MODERATELY RAPID COOLING STORED 22 DAYS	VERY RAPID COOLING STORED 20 DAYS
Plasma Hgb. (mg./100 Ml.)	39	50	79
% Hemolysis in 0.63% NaCl	4	3	10
A.T.P. mg./ml. Red cells	6.4	7.2	6.9

The specimens of blood used in the experiment reported above are the same used in the in vivo studies shown in Fig. 3.

Posttransfusion Survival Studies.—Much more pronounced differences between units of blood cooled at various rates are revealed by posttransfusion survival studies. Fig. 2 represents the effect of slow cooling and very rapid cooling on two units of blood obtained from the same donor and transfused immediately and after two and three weeks of storage into compatible recipients. Survival was measured with radioactive sodium chromate tagging. Very rapid cooling results in a rapid loss of the blood transfused within 24 hours of collection or after two weeks of storage. After 3 weeks of storage the survival rates are similar.

In another typical experiment, represented in Fig. 3, three units of blood were obtained from the same donor. The blood was collected through the metal coil, maintained at various temperatures, so as to obtain slow cooling, moderately

rapid cooling, and very rapid cooling in succession. Under each of these conditions approximately 160 ml. of blood was obtained in three standard glass containers.

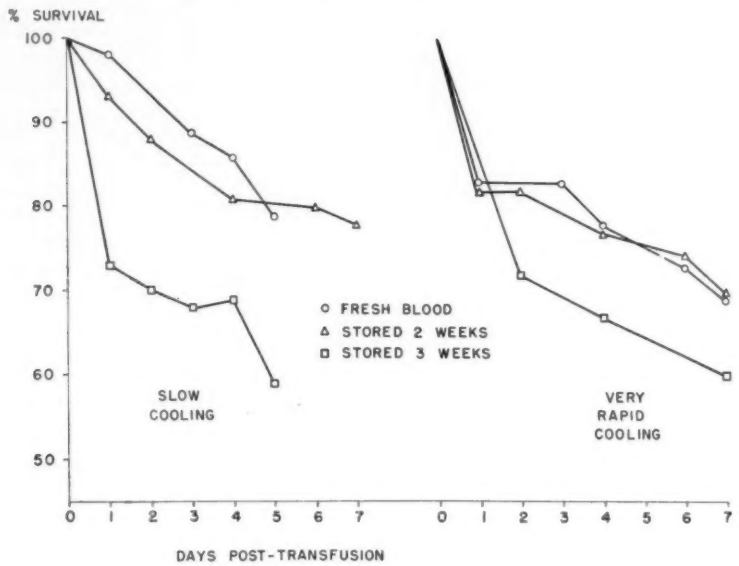

Fig. 2.—Effect of rate of cooling on posttransfusion survival of red cells tagged with $Na_2Cr^{51}O_4$.

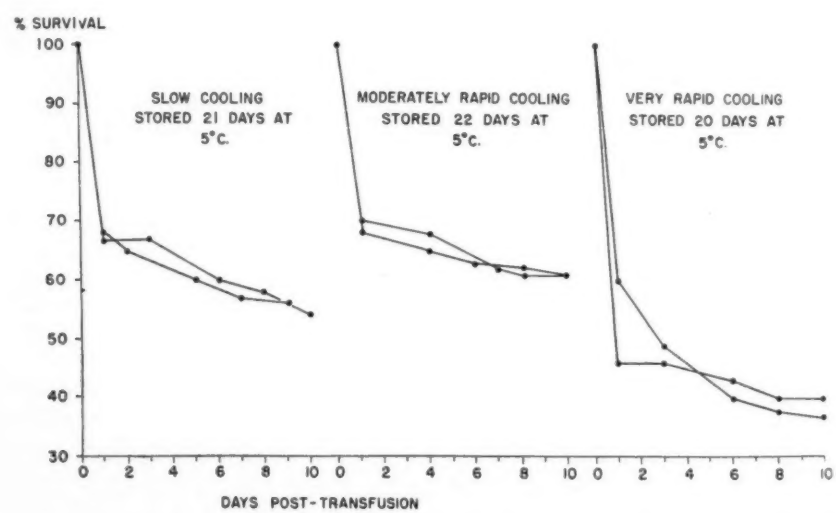

Fig. 3.—Effect of rate of cooling on posttransfusion survival of red cells tagged with $Na_2Cr^{51}O_4$.

The in vitro tests revealed relatively minor differences between the three types of cooling (Table I). At the end of approximately three weeks of storage at 5° C., each of the three units of blood was transfused into two compatible recipients. There is a slight difference in the survival rate between the red cell slowly cooled and those cooled at a moderately rapid rate, the latter surviving somewhat better after 22 days of storage. However, the blood

very rapidly cooled and stored for 20 days shows a very rapid disappearance of red cells in the first 24 hours posttransfusion, and thereafter the loss from circulation is much more rapid than noted with either slow or moderately rapid cooling.

Survival studies were also carried out on random specimens of blood collected in the conventional glass containers and transfused at various intervals of time. Fig. 4 shows the result of an experiment in which blood was stored for four weeks. The red cells of the blood very rapidly cooled disappeared at a rate much faster than the red cells of blood cooled at a moderately rapid rate.

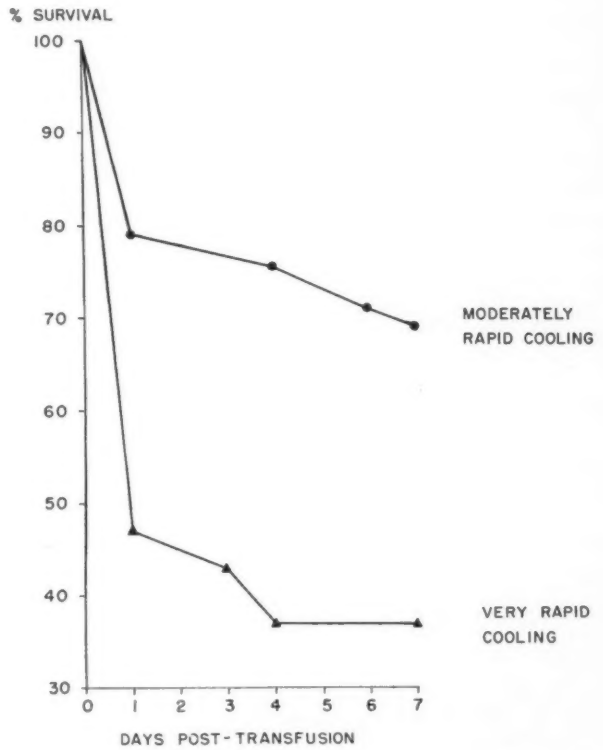

Fig. 4.—Effect of rate of cooling on posttransfusion survival of red cells tagged with $Na_2Cr^{51}O_4$.

Several experiments of similar nature have shown that invariably blood very rapidly cooled shows a posttransfusion survival poorer than blood cooled slowly or at a moderately rapid rate, although the difference is of varying magnitude in various experiments.

The effect of cooling of blood at a constant rate of approximately 10° C. per hour from 37° C. to storage temperature was determined. This rate of cooling did not improve the preservation of red blood cells.

DISCUSSION

Glenny and Stevens,[3] van Heyningen,[4] and McFarlane[5] have shown that red blood cells may be sensitized by bacterial toxins so that, when rapidly

cooled, hemolysis will occur. Lovelock[6] observed that red cells exposed to sodium chloride in concentrations stronger than 0.8 M may be sensitized in a similar manner, so that sudden cooling causes rapid and severe hemolysis. This sensitization is attributed to removal of lecithin from the cell, with resulting damage to the cell membrane.

The mechanism of this damage is related to the brittleness of the cell membrane, after loss of cementing phospholipids, under the stress of thermal contraction.[7]

Lovelock points out that in cells sensitized in the manner noted the factor which is most important in inducing hemolysis is not the rate of cooling but the range over which cooling occurs. In our experiments the rapid cooling did not seem to affect the glycolytic activity of the red cell but not infrequently altered the cell permeability, suggesting a damage to the cell membrane. This damage appears definitely related to the rapidity of cooling, rather than to the range over which the cells are cooled (37 to 5-1° C.). It is to be noted, however, that Lovelock's observations were all made on in vitro tests, whereas most of our observations are based on posttransfusion survival studies.

The optimal rate of cooling of red cells for purpose of transfusion has not been determined. However, in our experience cooling from 37° C. to a temperature of 5 to 1° C. in a period of one-half to one hour appears to be compatible with good red cell preservation.

CONCLUSIONS

Very rapid cooling of blood during collection damages red cells, definitely reducing their posttransfusion survival. This damage is probably due to a lesion of the cell membrane.

Moderately rapid cooling (to a temperature of 5 to 1° C. in a period of one-half to one hour) is compatible with good posttransfusion survival. This rate of cooling appears to give results somewhat more satisfactory than those obtained with slower rates, prevalent in the practice of blood banks.

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AGAR ELECTROPHORESIS OF SERUM PROTEINS ON CELLOPHANE AND POLYESTER FILMS

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IN EARLIER reports,[1, 2] I have described briefly a technique of agar electrophoresis for the separation of serum proteins. Since then a new technique has been developed for carrying out agar electrophoresis on cellophane and polyester films which has resulted in the development of a simple and quick method for evaluating the serum protein patterns by densitometric and elution analysis and for radioactive counting. Because this differs in some important characteristics from the procedure of Bussard and Perrin,[3] in the present paper a description of this technique and procedure is given.

MATERIALS AND METHODS

Agar.—Agar fine powder (B.D.H.) is used exclusively without subjecting it to any purification process, as it does not contain colored impurities to interfere with photometric analysis. The buffered agar gel is prepared by mixing equal volumes of the 2 per cent agar and 0.10 ionic strength Veronal-acetate buffer of pH 8.6 and it is used as a medium for electrophoresis. This is always kept at 50° to 60° C. before use.

Buffer.—Veronal acetate buffer pH 8.6, ionic strength 0.05, is used for each electrophoresis run. A stock solution of the buffer containing 29.43 Gm. of Veronal sodium, 19.43 Gm. sodium acetate trihydrate, and 180 ml. of N/10 HCl and made up to three liters is prepared. The stock solution is diluted 1:1 with distilled water to prepare the buffer of 0.05 ionic strength.

Dye Solution.—(Amido black 10B): The dye solution used for staining the protein components after electrophoresis is prepared by dissolving 5 Gm. of Amido black 10B in one liter of the solvent mixture of the composition, methanol-acetic acid-water (40:10:50).

Agar Plate Unit.—A schematic diagram of the unit is shown in Fig. 1.

Two pieces of plate glass (2 mm. thickness) cut into 30 cm. length and 5.2 cm. width are used, one for supporting the film with the buffered-agar gel and the other to be used as cover. Two frames of marginal width of 0.9 cm. are cut from a "Perspex" sheet (1.2 mm. thickness), the outer dimensions being exactly the same as the two plate glasses. Thin sheets of cellophane (0.0009 inch thickness) or Mylar polyester film† (0.00025 inch thickness), cut to the size of the plate glass, are used as support for the agar gel. The cellophane sheet is first wetted with water and placed over the plate glass. The moisture is removed by blotting with a filter paper and the sheet is pressed gently and evenly on the plate so as to obtain a smooth surface without the formation of wrinkles. The "Perspex" frame is placed on the cellophane. Two Whatman No. 3 paper strips cut to the size 8 cm. × 4 cm. are inserted at each end between the edges of the frame and the plate glass containing the cellophane. The frame and the glass plate with the paper strips are kept well clamped by means of spring clips and placed on a suitable support, arranged for proper leveling. Ten milliliters of the buffered agar gel (1 per cent; pH 8.6; ionic strength

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†Manufactured by E. I. du Pont de Nemours & Co. (Inc), Wilmington 98, Del.

0.05) while still warm is layered carefully with 10 ml. pipette on the cellophane into the space provided by the frame. The gel is allowed to cool at room temperature. Another "Perspex" frame is placed over the one already kept on the plate glass. After the agar gel layer is set, the plate glass with the frames is placed on the lips of the two electrode vessels of the Kelab paper electrophoresis equipment or any other suitable electrode vessels with the ends of the filter paper strips at each end dipping into the buffer solution (about 300 ml. of Veronal-acetate buffer, pH 8.6, ionic strength 0.05 in each vessel) in the electrode vessels. The distance between the buffer level and the agar plate is adjusted to about 1 cm.

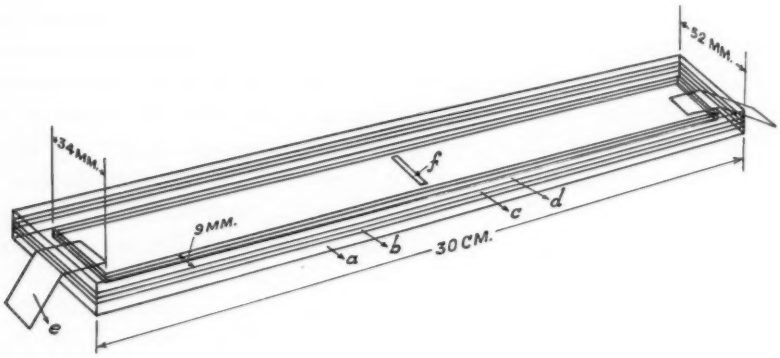

Fig. 1.—Schematic diagram of the agar plate unit. *a*, Plate glass to support the agar gel. *b*, "Perspex" frame enclosing the agar gel layered on cellophane or polyester film. *c*, "Perspex" frame as support for the cover glass. *d*, Plate glass cover. *e*, Whatman No. 3 filter paper strip. *f*, Position of filter paper strip for sample insertion.

Application of Sample.—The serum sample to be measured is carefully drawn into a 10 μl micropipette. The sample is streaked evenly on a small strip of Whatman No. 1 filter paper (20 × 2 mm.). The paper strip containing the serum is placed at the center of the agar gel layer perpendicular to the long axis of the plate without disturbing the surface of the gel, leaving space of about 2 mm. on each side of the gel layer. This technique of paper strip application ensures separation of the protein components into sharp zones.

Electrophoresis Run.—The power supply is adjusted to 200 or 300 volts. A voltage of 300 volts, with the setup used in these investigations, usually furnishes a current of 8 to 9.5 Ma., giving adequate separation in approximately 3 hours. If a lower voltage of 200 is used, the duration of the run is increased to 4 hours. The current is never allowed to exceed 10 Ma. Optimum separation is usually obtained in a run of between 3 to 5 hours.

Drying.—After completion of the electrophoresis, the top plate glass and the top "Perspex" frame are removed carefully without the drops of moisture condensed on the bottom surface of the plate falling onto the surface of the gel. The plate glass containing the agar gel with the other Perspex frame is removed from the electrode vessels. The two filter paper strips at both ends are detached from the gel layer. The small filter paper of serum is carefully removed from the surface of the gel by means of forceps without disturbing the surface of the gel. The plate glass supporting the gel and the "Perspex" frame are kept intact by rubber bands at the two ends of the plate and kept overnight (12 to 16 hours) at room temperature for drying. The time of drying can be reduced by keeping a fan running near the plate.

Staining.—After drying, the protein components separated on the agar gel are stained as follows:

The dry agar plate is immersed for about 30 minutes in the dye bath contained in a tall jar of one liter capacity. It is then removed from the dye bath and washed with a previously used methanol acetic acid (90 parts of methanol and 10 parts of glacial acetic acid) solvent in an enameled tray for about 5 minutes followed by a second wash in a fresh solvent

mixture. After the second wash, the cellophane can be peeled off from the plate glass and washed with fresh solvent for 30 to 45 minutes and dried at room temperature. By this procedure the protein components show as blue bands against clear and transparent background, without any free dye adsorbed on the agar layer. Similar procedure can be adopted for the Mylar polyester film. The time taken for washing of the dye, in this case, is short, however (3 to 4 minutes).

RESULTS AND DISCUSSION

The agar electrophoresis patterns of sera obtained on cellophane and Mylar polyester films are illustrated in Fig. 2. The technique affords a simple method of eluting the components separated on the gel by cutting the film into small strips at right angles to the direction of separation, as in the case of paper electropherograms, and extracting the components by suitable methods.

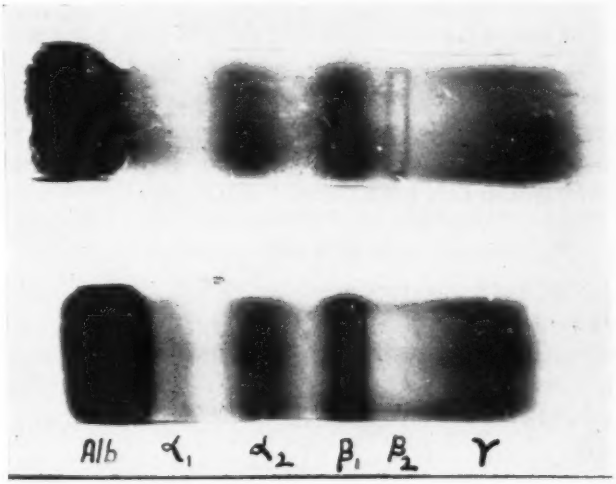

Fig. 2.—Agar electrophoresis of normal serum proteins obtained on cellophane and Mylar polyester films. *A*, Mylar polyester film. *B*, Cellophane.

The concentration of the substances can be determined by the usual methods of analysis. In the case of proteins stained with the dye, the concentration of the proteins can be determined by colorimetry of the blue color extracted by N/20 sodium hydroxide. The dye can be easily eluted from the cut film strips. The agar film can be peeled off from the polyester film, being water repellent, after drying and cut for elution and colorimetric estimation of the dye bound to the proteins. The same film can be used again for subsequent experiments. The electropherograms obtained on these films can be used for densitometric evaluation of the patterns by the scanning instruments used for direct photometry of paper electropherograms. As cellophane sheets are readily available and less expensive, and the electropherograms can be preserved for future reference, it is more convenient to carry out the routine analysis of serum proteins on cellophane instead of glass. In addition, the technique is useful for investigations of radioactive compounds, as the radioactivity of the substances separated on the thin film of agar can be easily determined, as the

self absorption of the thin films will not be as high as paper. It can be used in any continuous radioactive scanning device used for paper strips. As agar does not exhibit any absorption in ultraviolet light, the technique can be employed for the separation and quantitative determination of ultraviolet light-absorbing substances by ultraviolet photometry.

The technique affords the advantage over paper electrophoretic method of shorter experimental time, easier staining procedure, transparency of the dry agar film which facilitates photometric measurements, absence of trailing of proteins, and very small resistance offered by the gel to the movement of the proteins. Furthermore, the defects inherent in paper electrophoresis, namely, the anisotropy due to fibers and the reticular structure of the paper which makes the protein follow a tortuous path in its passage through the paper, thereby necessitating the introduction of a factor for determining the mobilities, are not present in the agar electrophoresis technique.

One of the important advantages of agar gel over the starch and paper as supporting media is that agar gel has a very low supporting medium–liquid ratio, which is an important factor in increasing the capacity of the system, without offering much resistance to the movement of large size molecules. The other supporting media used in electrophoresis, namely, paper and starch, occupy half or more than half of the total volume.

SUMMARY

A technique of agar electrophoresis of serum proteins on cellophane and polyester films is described, which possesses the advantages of simplicity, better resolving power without adsorption, simplicity in staining procedure, and easy adaptability to densitometric measurements, elution, and radioactive tracer analysis. It is suitable for routine analysis of serum protein fractions in any clinical laboratory.

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PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

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ABSTRACTS

1. THE EFFECT OF DEPO-NORTESTONATE ON THE NITROGEN BALANCE IN SUBTOTAL GASTRECTOMIZED PATIENTS

WILLIAM E. ABBOTT, M.D., HARVEY KRIEGER, M.D. (BY INVITATION), JOHN BRADSHAW, M.D. (BY INVITATION), AND STANLEY LEVEY, PH.D. (BY INVITATION), CLEVELAND, OHIO

Earlier we reported that the nitrogen deficit following surgical procedures could be negated or minimized by administering a regimen high in calories and nitrogen. This regimen depended on the use of a fat emulsion which could be given intravenously as a caloric supplement, and since it is not yet readily available it was decided to determine if similar results might be obtained by giving regimens adequate in nitrogen but containing only modest amounts of calories and by employing the anabolic hormone Depo-Nortestonate.

Metabolic balance studies were carried out using three groups of subtotally gastrectomized patients. Group I consisted of three male and three female patients who were maintained on intravenous regimens which were high in both calories (35 to 60 cal. per Kg. per day) and nitrogen (0.2 to 0.3 Gm. per Kg. per day). Group II consisted of three male and three female patients who were given an adequate nitrogen intake but only a modest caloric intake (20-25 cal. per Kg. per day), and Group III consisted of two male and three female patients who received essentially the same intravenous regimen as did Group II and who were given 250 mg. of Depo-Nortestonate one or two days preoperatively. The male patients of Group I had an average five-day (day of operation plus the first four postoperative days) cumulative nitrogen deficit of 10 Gm. and the females of this group had an average positive nitrogen balance of 0.5 Gm. The males in Group II had an average cumulative nitrogen deficit of 37 Gm., and an average cumulative deficit of 11 Gm. was shown by the females. In Group III (the patients who received the anabolic hormone) the males had an average five-day cumulative nitrogen deficit of 16 Gm. and the females showed an average positive nitrogen balance of 6 Gm. This work indicates that the postoperative nitrogen deficit can be reduced by administering either a regimen high in nitrogen and calories or by giving adequate nitrogen with only a modest caloric intake and an anabolic hormone such as Depo-Nortestonate.

2. SENSITIVITY OF HUMAN SUBJECTS TO BRUCELLA ENDOTOXIN AND ITS SIGNIFICANCE IN THE PATHOGENESIS OF BRUCELLOSIS

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(INTRODUCED BY WENDELL H. HALL, M.D.)

The pathogenesis of the manifestations of brucellosis has been investigated in this clinic for a number of years. Observations of patients have indicated that acquired sensitivity to cellular components of the brucella cells plays a significant role in the illness in both acute and chronic brucellosis. The present investigation is concerned with the response of patients who had objective evidence of having been infected by brucella in the past to a standardized preparation of brucella endotoxin, in contrast to the response of normal controls. When 50 to 100 meg. of endotoxin was given intravenously to the latter group of 15 persons, no febrile or systemic reactions were detected. On the other hand, in a group of 25 patients having positive intradermal tests to brucella antigen, reaction followed the injection of endotoxin ranging from 1 to 100 meg. Fourteen of the 25 patients with positive skin tests presented no historical evidence of an infection in the past; they had a low titer of brucella agglutinins; and only 8 of these individuals experienced any reaction to endotoxin. But most significantly, in the 11 individuals convalescing from acute brucellosis, or with a definite history of active disease in the past, 10 experienced fever and systemic reactions. The reactions in this latter group were more severe and were provoked with smaller doses of endotoxin. In both groups, the severity of the reaction to endotoxin showed suggestive correlation with the degree of dermal hypersensitivity. These data lend further support to the thesis that hypersensitivity to brucellae is of considerable significance in the pathogenesis of the illness.

3. PRIMARY PULMONARY HYPERTENSION

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(INTRODUCED BY KENNETH KOHLSTAEDT, M.D.)

The principal physical findings of increased pulmonary blood flow are an accentuated pulmonary second sound, a diastolic left parasternal shock, a systolic and/or diastolic murmur, and right ventricular hypertrophy. When these are "paradoxically combined" with the roentgen features of scanty lung vasculature, which usually indicates diminished pulmonary blood flow, the diagnosis of primary pulmonary hypertension should be suspected.

The symptoms, although disabling, are paroxysmal and evanescent so that the patient is often considered neurotic. Dyspnea of a severity disproportionate to the effort which produces it is a nearly invariable feature of the syndrome, but it may be accompanied by effort syncope and effort angina.

The three young adult women studied were symptomless at rest but incapacitated by dyspnea with slight exertion; two had effort syncope and one effort angina. Right heart catheterization revealed resting pulmonary artery pressures of 102/42, 72/34, and 70/27; the latter two pressures rose sharply to 91/42 and 106/49 with exercise. The highest resting cardiac output was 3.14 L./min., and the greatest exercise output was only 4.17 L./min. No left to right shunt was found, but in one patient the foramen ovale was traversed

with the catheter. The left atrial pressure was −1.4 mm. Hg while the right was −2.7 mm. Hg. That cyanosis can be produced through such a foramen was demonstrated during exercise at which time the right atrial pressure rose to 3.5 mm. Hg and there was prompt arterial oxygen undersaturation uncorrected by the administration of oxygen.

No adverse effects were noted during catheterization even with exercise, contrary to the experience of others. Diagnostic right ventricular hypertrophy was present on all electrocardiograms.

A necessary factor in the correct final diagnosis of the syndrome is the absence of lung disease. No evidence of such was found in these cases, and complete pulmonary function tests revealed no physiologic deviation from normal.

4. THE EXPERIMENTAL USE OF A HEART-LUNG MACHINE

Bernard A. Bercu, M.D., and Gerald Diettert, M.D., St. Louis, Mo.

(Introduced by John R. Smith, M.D.)

Although various heart-lung machines are now being utilized to support the circulation during cardiac surgery, little information is available in the literature to delineate the physiologic principles which must be followed if survival of the patient is to be expected. The authors propose to describe these principles and their application based upon a series of animal studies using dogs. A pump-oxygenator apparatus modified from that described by DeWall and Lillehei has been used. The oxygenator is of the bubble type. Sigmamotor pumps are employed for the arterial, venous, and coronary sinus circulations. Arterialized blood passes through a nylon mesh filter before being pumped to the animal. The filter is so constructed that it also functions as a bubble trap and is an added safety factor in preventing the introduction of air into the circulation.

The principles which seem to be the most important for the proper use of the machine are as follows:

1. Flow rates of 60 to 70 ml./Kg./min. were considered more physiologic than those based upon the azygos flow principle.

2. An internal circulation of the pump-oxygenator, used prior to perfusion of the animal, is essential to determine the exact level of blood in the arterial reservoir while all of the parts of the apparatus are filled with blood and the pumps are running. This procedure permits exact control of the animal's blood volume during the period of perfusion.

3. Once occlusion of the patient's circulation has been effected and the arterial and venous flows are stabilized, they are not altered until after the occluding ligatures are released. It has been found that any decrease in venous return to the pump is usually due to blood loss and should be compensated by adding blood to the reservoir.

4. Once circulatory occlusion has been terminated, a period of continued perfusion with the pump while part of the circulation is passing through the heart is important to allow the circulation to stabilize.

5. The arterial pressure of the animal must be carefully maintained once the machine is stopped.

5. THE USE OF CELERY AS THE BASIS OF A RANDOM DIET IN A PROGRAM FOR WEIGHT REDUCTION

CLARENCE BERNSTEIN, M.D., S. D. KLOTZ, M.D. (BY INVITATION), TYBEE W. KAHN, B.S. (BY INVITATION), AND WILLIAM G. O'REGAN (BY INVITATION), ORLANDO, FLA.

The need for a readily accomplished weight reduction program is too evident to require much exposition. In the past programs for restricted caloric intake, much has been desired in the way of giving some comfort to the patient who is on such a regimen. Ideally such an individual would like to have a reasonably free choice of food, to feel that he was eating enough, and to have some degree of ingestion satisfaction on the program. Celery appeared to be an ideal food for this purpose since it was abundantly available. It is natural rather than synthetic and it is chewable, mouth filling, and swallowable. It has a pleasant taste and supplies effective gastrointestinal bulk furnishing something on which peristalsis can act and be quieted.

Two series of these patients were studied using celery as an adjunctive filler material in the dietary, and a random choice of foods to maintain table comfort. Studies were made employing in the same manner a methyl-cellulose hydrophilic tablet and a placebo tablet (a relatively inert, nonswelling material). All groups lost weight. The methyl-cellulose tablets apparently possess slightly more effectiveness in the one study in which it was used. The other properties of celery seem to outweigh the slight possible advantage of the methyl-cellulose tablets and these properties are pointed out. Statistical evaluations indicate the weight loss to be significant. Certain observations made on these groups of patients will be reported.

6. INHIBITORY EFFECT OF "ISOCHOLESTEROL" ON THE ABSORPTION OF CHOLESTEROL

MAURICE M. BEST, M.D., AND CHARLES H. DUNCAN, M.D. (BY INVITATION), LOUISVILLE, KY.

The effects on the intestinal absorption of cholesterol of substances related chemically to cholesterol have been under study. The present report deals with "isocholesterol," the designation that has been applied to a mixture of C_{30} compounds occurring in wool fat. The major constituents of the mixture are reported to be lanosterol ($C_{30}H_{49}OH$) and dihydrolanosterol. Also present are agnosterol ($C_{30}H_{47}OH$) and its dihydro derivative.

Male albino rats of approximately 300 grams were maintained for 2 weeks on the following experimental diets: (a) low-cholesterol, consisting of Purina Rabbit Chow plus 5 per cent cottonseed oil, (b) high-cholesterol, consisting of diet "a" plus 1 per cent cholesterol, and (c) cholesterol-"isocholesterol," consisting of diet "b" plus 5 per cent "isocholesterol." At the end of 14 days the animals were sacrificed and the cholesterol concentration of serum and liver was determined.

The mean serum total cholesterol concentrations expressed in mg./100 ml. were as follows: (a) low-cholesterol diet 72 ± 5, (b) high-cholesterol diet 82 ± 12, and (c) cholesterol-"isocholesterol" diet 68 ± 5. The mean liver cholesterol concentrations expressed as mg./100 grams liver (wet weight) were 303 ± 6, 1,224 ± 388 and 342 ± 32, respectively.

A similar study utilized rats rendered hypothyroid by the prior administration of I^{131}. The mean serum total cholesterol levels following 14 days'

administration of the low-cholesterol, high-cholesterol, and cholesterol-"isocholesterol" diets were 85 ± 13, 119 ± 24, and 98 ± 6, respectively. The mean liver concentrations were 262 ± 27, 1,625 ± 296, and 355 ± 67.

These results indicate that the addition of "isocholesterol" to a high-cholesterol diet prevents the expected accumulation of cholesterol in serum and liver. This effect is presumed to be due to interference with cholesterol absorption by one or more of the constituents of "isocholesterol."

The effect of "isocholesterol" on human subjects with hypercholesterolemia has been studied. The administration of 9 to 18 Gm. per day without dietary restriction resulted in a reduction in serum total cholesterol in 5 of 6 patients.

7. URINARY EXCRETION OF VITAMIN $B_{12}Co^{60}$ IN MYXEDEMA

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CHICAGO, ILL., AND HINES, ILL.

A man with marked megaloblastic anemia and myxedema aroused our interest in possible dynamic relationships between these conditions. He had free gastric acidity after histamine. Gastroscopy was normal. There was no evidence of hepatic or intestinal disease. Response to vitamin B_{12} therapy was suboptimal. One or 2.0 μg $B_{12}Co^{60}$ without (*C*) or with one to three U.S.P. units intrinsic factor concentrate (*IFC*) orally were followed by 1.0 mg. B_{12} parenterally, and percentage $B_{12}Co^{60}$ in 24-hour urine was measured. Four sets of tests (*C* and *IFC*) during nine months of thyroid therapy (mostly 120 mg./day) showed low (under 4 per cent with 1.0 μg) or occasionally low intermediate (under 6 per cent) excretions. *C* remained low after four days of tetracycline. One milligram B_{12} parenterally was repeated at 24 and 48 hours during one set. Seventy-two-hour excretions were: *C* = 9.6 per cent, *IFC* = 3.3 per cent. Two aspirates (25 and 75 ml.) of patient's gastric juice (*PGJ*) showed insignificant intrinsic factor (*IF*) activity in patients with pernicious anemia (*PA*). Three aspirates *PGJ* (20 to 62 ml.) were tested for inhibition of *IFC* in *PAS*. One indicated and another suggested inhibition.

Two other untreated myxedemas showed low or low intermediate *C* and *IFC* excretions. One received 4 days chlortetracycline, and *IFC* test remained low. Three other myxedemas, one under treatment, showed normal excretions (over 8 per cent with 1.0 μg).

Megaloblastic anemia in hypothyroidism is rare and has been attributed to chance. Malabsorption type $B_{12}Co^{60}$ tests in three of six selected hypothyroids suggest that in some cases absorption of B_{12} is hampered by intestinal block or by intraluminal inactivation of intrinsic factor or vitamin B_{12}. Poor *IF* activity of *PGJ* indicates either a concomitant deficiency of *IF* secretion, or, more credible, an inactivating substance in *PGJ*. Inhibition was demonstrated in one of three aspirates. This, plus paradoxical and prolonged 72-hour excretions, suggests variable regurgitation from the intestine of an inhibitor which in minimal amounts delays absorption. This is probably nonbacterial, as antibiotics failed to correct malabsorption. Consistent malabsorption despite prolonged therapy could indicate inadequate dosage of thyroid, irreversible change from hypothyroidism, or a hypothyroid state secondary to intestinal malfunction.

8. MORPHOLOGIC CHANGES IN SPLENIC SINUSOIDS IN IDIOPATHIC THROMBOCYTOPENIC PURPURA

ROBERT M. BIRD, M.D., WALTER JOEL, M.D. (BY INVITATION), AND TED CLEMENS, JR., M.D. (BY INVITATION), OKLAHOMA CITY, OKLA.

Conventional staining of splenic tissue has been unrewarding in differentiating various splenic syndromes. This prompted the use of connective tissue stains. The following were used: van Gieson's picric acid fuchsin, Wilder's silver impregnation, periodic acid Schiff (McManus), combined neutral and acid polysaccharides (Rhinehart, Abul-Haj), azan (Heidenhain's aniline blue), and Masson's trichrome (Goldner's modification). The material studied consisted of fifty-five spleens: fifteen from patients with no blood dyscrasias; twenty-eight removed because of a variety of hematological disorders; and twelve from patients whose illness was diagnosed clinically as idiopathic thrombocytopenic purpura. The variations in the latter twelve compared with the others form the basis of this report. In this purpura group, the follicles were frequently increased in size and number and showed conspicuous marginal zones. Especially prominent were germinal centers. Sinuses were often dilated and devoid of blood. Although we share with others the opinion that such alterations are noteworthy, they proved to be nonspecific, varying markedly with age, and hence unreliable in the differentiation of splenic syndromes. The P.A.S. stain for neutral polysaccharides, however, brought out a prominence of the mebrana propria of the sinusoids which was a more reliable differential criterion. The combined stain made these differential features even more striking. The changes were characterized by the appearance of beads and/or short, regularly spaced, but interrupted fibers. These fibers appeared to encircle the sinusoids. Viewed on end they resembled beads while longitudinal cuts gave them the appearance of rungs of a ladder. Having observed this, we reviewed all specimens without knowledge of the diagnosis. A similar configuration was noted in nearly all, but the distinguishing feature was the degree of prominence of the membrana propria. Nine of the twelve spleens were correctly identified as idiopathic thrombocytopenic purpura. In no instance was a mistaken identification made in spleens from other conditions including other clinical types of purpura. This appeared to emphasize the differential value of the P.A.S. and combined stains and arouses the speculation that idiopathic thrombocytopenic purpura may be another of the "collagen diseases," in this case involving the sinusoids rather than arteries.

9. THE EFFECTS OF DIGITALIS GLYCOSIDES ON THE ELECTROLYTE BALANCE OF THE MYOCARDIUM: ALTERATION OF THESE EFFECTS BY GLUCOSE AND INSULIN AND POTASSIUM CHLORIDE

JOHN R. BLACKMON, M.D. (BY INVITATION), LOUIS GILLESPIE, M.D. (BY INVITATION), ROBERT M. BERNE, M.D. (BY INVITATION), AND HERMAN K. HELLERSTEIN, M.D., CLEVELAND, OHIO

The effects of two digitalis glycosides on the sodium and potassium balance of the myocardium and the alterations of these effects by the administration of glucose and insulin or potassium chloride were studied in twenty-eight dogs. In open-chest dogs a Morawitz cannula was placed in the coronary sinus and coronary sinus blood flow recorded. Coronary sinus and arterial samples were collected simultaneously. Serial electrocardiograms and aortic pressures were recorded.

In each of nine dogs "toxic" doses of Acetyl Strophanthidin or K-Strophosid produced a negative myocardial potassium balance during the first fifteen to twenty-five minutes, followed by a positive potassium balance. However, a cumulative potassium debt was present throughout each experiment, and although this debt was being repaid during the latter part of each experiment, serial electrocardiograms showed progressive deterioration, terminating in bizarre arrhythmias typical of digitalis intoxication or ventricular fibrillation. "Therapeutic" doses of these glycosides caused a significant release of potassium from the myocardium of lesser magnitude. There were no significant changes in serum sodium in this series. A consistent rise in arterial potassium throughout each experiment also points to an extracardiac release of potassium.

In seventeen dogs glucose and insulin were administered prior to, simultaneously with, or following the digitalis glycoside. Glucose and insulin produced a rapid deterioration of the electrocardiogram and a marked shortening of the survival time. This effect was greatest when given near the peak of digitalis intoxication, and less deleterious when the glucose and insulin were given together with or prior to the digitalis glycoside.

The effects of slow intravenous infusions of potassium chloride prior to administration of digitalis glycosides were studied in five dogs. The myocardial potassium debt was of smaller magnitude, and the electrocardiographic changes were less striking, often returning to normal.

CONCLUSIONS

"Toxic" doses of digitalis caused a rapid release of myocardial potassium and slower repayment of the potassium debt, at which time there was progressive hemodynamic and electrocardiographic deterioration. "Therapeutic" doses also caused a significant but lesser potassium release. Potassium chloride administration protected the myocardium, while glucose and insulin accelerated the digitalis intoxication. Our studies suggest that the magnitude of digitalis intoxication is related to both the extracellular potassium concentration and the *state* of the intracellular potassium (bound or ionic) rather than to the intra- or extracellular potassium concentration alone.

10. A SLIDE RULE FOR THE EVALUATION OF PERCENTAGE DIFFERENCES

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(INTRODUCED BY WILLIAM B. BEAN, M.D.)

This paper describes a slide rule for the evaluation of percentage differences between two series of observations. The number of cases in the smaller of the two series to be compared, N_A, is represented on scale α. The percentage difference in the incidence of the phenomenon under study, Δp, is represented on the slide (scale β). The percentage incidence of the phenomenon in both series combined, p_0, is represented on scale γ.*

The use of the slide rule may be illustrated by means of a simple example: The mortality of 10,000 cases of Z disease was 10 per cent. A physician treats 100 patients with this disease by a new method; the mortality is 4 per cent. Is the success of his treatment due to chance alone? To answer this question with the slide rule one proceeds as follows: (1) Find the value

*If p_0 is larger than 50, an alternate value $q_0 = 100 - p_0$ should be used.

100 on scale. (2) Move the slide until the numeral 1 on scale β coincides with the numeral 100 on scale α. (3) Move the cursor until its hairline coincides with $\Delta p = 6$ $(= 10 - 4)$ on scale β. (4) Read the value of p_0' on scale γ which coincides with the hairline of the cursor; p_0' is found to be about 5.5. (5) Find the value of $p_0 = (4 + 1{,}000)/(100 + 10{,}000) \times 100 \approx 10$ on scale γ.

The relation of p_0' to p_0 determines whether or not the difference between the two series is "statistically significant." The slide rule which accompanies this abstract is constructed to differentiate levels of significance which are higher than $P = 0.01$ from those which are lower than $P = 0.01$.* If p_0' is greater than p_0 (that is if p_0' lies to the right of p_0) the difference Δp between the two series is more significant than for $P = 0.01$; if p_0' is less than p_0 (that is if p_0' lies to the left of p_0) Δp is less significant than $P = 0.01$; if $p_0' = p_0$ the level of significance is $P = 0.01$. In our example p_0' is smaller than p_0; therefore the difference between the two series is not significant at the level of $P = 0.01$.

Additional features of the slide rule as well as its limitations and the principles involved in its construction will be described in the discussion.

11. THE HEXOSAMINE CONTENT OF THE SERUM GLOBULINS IN NORMAL AND PATHOLOGIC SERA

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(INTRODUCED BY GORDON B. MYERS, M.D.)

Alterations in the hexosamine content of serum globulin fractions were studied in a variety of illnesses, with emphasis on rheumatic diseases, and following administration of anti-inflammatory hormones. Normal sera averaged 17.5 mg. per cent hexosamine in the alpha-1 globulin, 25.0 mg. per cent in the alpha-2, 17.4 mg. per cent in the beta, and 24.7 mg. per cent in the gamma globulin. The ratio of hexosamine to protein (× 100) was found to average 4.3 in the alpha-1, 4.1 in the alpha-2, 2.9 in the beta, and 1.8 in the gamma globulin. In both rheumatic and some nonrheumatic diseases the hexosamine content of the alpha-1 and alpha-2 globulins increased significantly, and the ratio of hexosamine to protein increased significantly in the alpha-1, but not in the alpha-2 globulin. Minimal changes occurred in the beta globulins. Sera with increased gamma globulin had an increased hexosamine content of that fraction with no change in ratio of hexosamine to protein. Anti-inflammatory hormones caused a fall in the hexosamine content of the alpha-1 and alpha-2 globulins, but a corresponding fall in hexosamine to protein ratio occurred only in the alpha-1.

The hexosamine content of the seromucoid fraction of Winzler was found to average 5.69 mg. per cent in normals, with a ratio of hexosamine to protein (× 100) of 7.20. The amount of hexosamine and the ratio of hexosamine to protein were increased in both rheumatic and nonrheumatic inflammatory diseases. Anti-inflammatory hormones caused a fall in the hexosamine content but a rise in the protein in the seromucoid in most instances, with the ratio of hexosamine to protein decreasing in all instances.

These observations are interpreted as indicating that in the alpha-1 globulins and the seromucoid there seems to be an increase in at least one component which is richer in hexosamine than the rest of the fraction, in a variety of illnesses, and that there is a fall in a hexosamine-rich component with anti-inflammatory hormone therapy.

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*Also, it is designed only for comparison of two series which differ in the number of cases by a facort or more than 20.

12. HODGKIN'S DISEASE OF THE BONE MARROW

WILLIAM H. BOND, M.D. (BY INVITATION), AND ROBERT J. ROHN, M.D., INDIANAPOLIS, IND.

Hodgkin's disease, a malignant granulomatous disorder of the reticuloendothelial system, is frequently associated with nonspecific alterations in marrow constituents and, on autopsy, examination may show patchy invasion of the bone marrow cavity.

Because of the syncytial nature of the pathologic process, the attendant fibrosis, or other unexplained causes, it is extremely rare to be able to demonstrate Reed-Sternberg cells on random needle aspiration of the marrow cavity.

Of the eighty patients with Hodgkin's disease seen in this clinic, three who had been diagnosed by standard fixed tissue techniques, lymph node biopsy, etc., demonstrated numerous abnormal reticulum cells of the Reed-Sternberg type in bone marrow aspiration biopsy material during the course of their disease.

The clinical features of these patients and their response to treatment are outlined. Photomicrographic demonstration of tissues prepared with hematoxylin and eosin stain, and Wright's stain will be presented to outline the detailed comparative cytological characteristics of these cells, and time-lapse phase contrast microcinematographic studies will be presented to show the motility behavior of these cells and, in one patient, the effect of CB-1348 upon the behavior of such cells. Motility patterns of other neoplastic cells will be shown for comparison.

13. THE ELECTROCARDIOGRAPHIC DIAGNOSIS OF VENTRICULAR HYPERTROPHY IN THE PRESENCE OF RIGHT BUNDLE BRANCH BLOCK

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All the Cincinnati General Hospital post-mortem records from the years 1950 to 1955, inclusive, were reviewed and all those with electrocardiographic (ECG) diagnoses of right bundle branch block (RBBB) subjected to analysis. It was stipulated that a 12-lead ECG must have been taken within three months of death, and all cases with myocardial infarction were discarded. During this 6-year period over 3,000 autopsies were performed and 49 cases fulfilling our criteria were obtained.

Post-mortem findings revealed: 4 instances of isolated right ventricular hypertrophy (RVH), 15 of isolated left ventricular hypertrophy (LVH), 22 of combined left and right ventricular hypertrophy (CVH), and 8 with hearts of normal measurements.

Study of the electrocardiograms in 28 cases of incomplete RBBB revealed 8 cases which fulfilled Barker and Valencia's criteria for coexisting RVH, i.e., right precordial $R' > 10$ mm. Of these, 2 cases had RVH, 1 LVH, 3 CVH, and 2 no hypertrophy at autopsy. One case met ECG criteria for LVH but had RVH at post mortem and 1 revealed CVH on the ECG but LVH at post mortem. The remaining 18 at autopsy showed LVH in 5, CVH in 11, and no hypertrophy in 2.

The 21 cases of complete RBBB revealed 3 with ECG evidence of associated RVH, i.e., $R' > 15$ mm. over the right precordium. At autopsy 1 had

RVH, 1 LVH, and 1 CVH. The remaining 18 had LVH in 7, CVH in 7, and no ventricular hypertrophy in 4.

These results indicate that in our series:

1. Anatomic LVH, either isolated or combined, was consistently masked on ECG by both complete and incomplete RBBB.

2. Those cases with electrocardiograms which met Barker and Valencia's criteria for coexisting RVH frequently, but not invariably, displayed RVH at post mortem.

3. In 49 cases of etiologically unselected RBBB, only 26 (53 per cent) showed anatomic evidence of either isolated or combined right ventricular hypertrophy.

14. RELIABILITY OF TESTS OF RAPID AND TOTAL EXPIRATION USING A LIGHTWEIGHT DIRECT WRITING SPIROMETER

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(Introduced by Robert A. Hettig, M.D.)

The frequency response of conventional spirometers has been critically evaluated by Stead and associates and they have given excellent ideas for improvements. A lightweight direct writing spirometer was constructed according to these recommendations so that the velocities and accelerations of the human breath could be studied.

The changes in the conventional spirometer were: (1) substituting a bell made of Fiberglas coated with plastic; (2) attaching the recording pen directly to the bell; (3) decreasing the volume of water inside the spirometer by inserting a closed plastic cylinder; and (4) allowing the spirometer bell to float freely. These modifications give the theoretical advantages of reduction of bell weight by one-half, avoidance of the errors inherent in an indirect recording system, and decrease of the irregular damping effect of water.

To test this new spirometer, 163 maximal midexpiratory flows (MMF), maximal one-half second volumes expired, and vital capacities were done in duplicate. The range of MMF was from 0.140 liter per second to 4.600 liters per second and the range of vital capacity was from 680 ml. to 4,400 ml. The reliability of these duplicate determinations was tested by statistical analysis. For the MMF, the reliability coefficient was 0.963, the regression was 0.94, and the standard error of the estimate was 0.226 liter per second. The volume expired in one-half second had a reliability coefficient of 0.979, a regression of 0.99, and a standard error of the estimate of 130 ml. The vital capacity had a reliability coefficient of 0.996, a regression of 1.00, and a standard error of the estimate of 82 ml.

Four nebulized bronchodilators were given to each of twenty patients and, with the use of the new spirometer and these tests, significant differences of values were obtained before and after administering the drugs. Following bronchodilators, there was observed an increase in vital capacity ($p < 0.01$), an increase in volume expired in one-half second ($p < 0.01$), and an increase in MMF ($p < 0.05$). With the use of the standard errors of the estimate, significant differences between individual values could be easily seen.

15. STUDIES OF THE FUNCTIONAL CAPACITY OF AUTOTRANSPLANTED KIDNEYS IN DOGS WITH COMPARATIVE DATA ON THE CONTRALATERAL (CONTROL) KIDNEYS

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(INTRODUCED BY CARL G. HARFORD, M.D.)

Progress in homotransplantation of human kidneys emphasizes the need to thoroughly assess the functional capacity of transplanted kidneys. Moreover, electrolyte excretion data from transplanted (therefore denervated) kidneys may aid in clarifying the disputed role of renal nerves in electrolyte transport. In an experimental approach to this problem, simultaneous functions of the separate kidneys of unanesthetized dogs with divided urinary bladders were measured serially. After control studies, one kidney in each animal was autotransplanted (five dogs) or denervated in situ (two dogs). Contralateral kidneys served as controls. Follow-up clearances were obtained up to six months after surgery.

GFR in all transplanted kidneys decreased markedly initially and thereafter increased, ultimately equaling control (contralateral) values in two animals. Regardless of level, GFR increased substantially in transplanted as well as control kidneys following ECF expansion with hypertonic NaCl, hypotonic NaCl, or synthetic ECF. Renal plasma flow initially decreased in transplanted and increased in control kidneys. Thereafter values approached their individual pretransplantation levels.

Concentrating ability of transplanted kidneys ($T^{e}_{H_2O}/C_{Cr}$) compared favorably with control kidneys, although the response to Pitressin was not always as prompt.

A relative sodium-losing state occurred in most transplanted kidneys. However, one animal did not manifest this abnormality, and in two others it ultimately disappeared. Similar defects were not apparent in nontransplanted denervated kidneys. Following ECF expansion, Na reabsorption increased with GFR in all kidneys, thereby establishing filtration dependence of Na reabsorption in denervated kidneys. During anesthesia, GFR consistently decreased only in control kidneys and changes in Na excretion correlated with alterations of filtered loads.

It is concluded that: (1) Autotransplantation of dog kidneys is associated with impairment of renal hemodynamics which is reversible even though contralateral clearance values may be elevated. (2) Permanent denervation is not associated with persisting gross defects in Na transport. (3) Anoxia sustained during transplantation may be causally related to the physiologic abnormalities.

16. TEMPORARY CIRCULATORY OCCLUSION: APPLICATION TO CARDIOVASCULAR DIAGNOSIS

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(INTRODUCED BY ERWIN LEVIN, M.D.)

Temporary unilateral pulmonary artery occlusion by means of a balloon-tipped cardiac catheter has provided valuable cardiorespiratory data in over 100 patients without significant adverse reaction. In another 30 patients,

angiocardiographic studies have been carried out successfully with instantaneous intracardiac release of large volumes of contrast material via an inflatable cuff (intracardiac angiography).

During performance of these procedures in the past three years, on occasion complete circulatory occlusion has been produced inadvertently, for periods up to 40 seconds. In at least five instances the balloon was inflated in the main pulmonary artery; in another case, the right pulmonary artery was occluded in a patient subsequently shown to have congenital absence of the left pulmonary artery. In each instance there was immediate relief of moderate distress with deflation of the balloon. (In dogs, relatively long periods of occlusion produced no permanent ill effects.)

Since inadvertent circulatory occlusion appeared to be so well tolerated, intentional temporary occlusion has been applied where indicated, as in the following case: In a 26-year-old man with sudden onset of rapidly progressive heart failure, a loud continuous murmur and thrill with a wide pulse pressure suggested a shunt from the aorta to pulmonary artery or right ventricle. Cardiac catheterization revealed arterialization of the outflow tract of the right ventricle. Angiocardiograms did not help in localizing the defect. Contrast material rapidly injected via a large retrograde aortic catheter was so rapidly dispersed by the blood flow that no visualization was obtained. Temporary circulatory occlusion was then produced by inflation of a balloon in the inferior vena cava and manual compression of the neck and arms; retrograde injection then revealed excellent visualization of a rupture of the right sinus of Valsalva into the right ventricle. This defect was confirmed at open heart surgery.

17. A COMPARISON OF SERUM INFLUENZA ANTIBODIES FOUND IN DOMESTIC ANIMALS WITH THOSE OCCURRING IN HUMAN ADULTS AND CHILDREN

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Demonstration of antibodies against St. Louis encephalitis virus in domestic fowl and animals served to clarify the epidemiology of this disease. Other than studies of swine influenza virus and observations on nonspecific inhibitors of the hemagglutinin phenomenon, little appears in the literature concerning naturally occurring influenza antibodies in domestic animals.

In 1945 to 1950, 1951, and 1955, we have studied the antihemagglutinin and complement-fixing antibodies in sera of domestic animals in comparison with those found in human sera. All sera were heat inactivated and those tested in 1955 were treated with R.D.E.

In an abstract submitted to this Society a year ago, and in an exhibit presented at the June, 1956, Meeting of the American Medical Association, we reported the difference between antibody patterns found in adults and those in children. A high percentage of adults born prior to 1930 show the presence of antibodies against swine, PR-8-A, and Lee Type B viruses, whereas

those born subsequent to 1945 rarely exhibit antibodies against these virus strains after the first four months of life.

Sera of normal mice, hamsters, rats, and ducks so far studied have not shown antibodies against influenza viruses. Sera of chicken embryos and newly hatched chickens are almost entirely free of influenza antibodies and this is largely true of older chickens raised in isolation. Sera of chickens from poultry dealers and from farms, that of rabbits (some from farms), and sera of dogs, guinea pigs, cattle, sheep, and hogs show patterns of antihemagglutinins and complement-fixing antibodies strikingly similar to those of children born in the same year as those animals and differing from those of adults.

This particularly concerns the absence or extremely low titers of antibodies to virus strains isolated prior to 1943, such as swine, PR-8-A, and, with some exceptions, Lee B, and the presence of antibodies against A-prime strains isolated since 1946, and recently occurring B strains. No antibodies against Type C virus were found in animals so far tested, although present in sera of human adults and children. Possible significance of these findings will be discussed.

18. A NEW SYNDROME: HEMOGLOBIN SC-THALASSEMIA, WITH ADDITIONAL OBSERVATIONS IN HEMOGLOBIN C THALASSEMIA

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In recent years the study of human hemoglobin has led to the definition of several new clinical syndromes. It has been predicted that additional entities will be described. We should like to report on a Negro family in which the father had C thalassemia and the mother sickle-cell trait. Their eleven children were found to have various combinations of these anomalies. Two were believed to represent the previously undescribed entity hemoglobin SC-thalassemia.

The father had a mild microcytic normochromic anemia, numerous target cells, and splenomegaly. Electrophoresis of his hemoglobin revealed 78 per cent C and 22 per cent A. Fetal hemoglobin was normal by the alkaline denaturation method. Erythrocyte survival studies indicated a shortened lifespan. With these findings it was concluded the father had the hemoglobin C trait plus the thalassemia trait. His sister had almost identical findings on electrophoresis and was thought to have the same abnormality. The mother showed uncomplicated sickle-cell trait.

The propositus and two siblings had SC hemoglobin. The propositus was first seen during an episode of bacterial pneumonia and had a mild hemolytic crisis with splenomegaly. Subsequent observations revealed normocytic, normochromic erythrocytes without anemia, numerous target cells, moderately decreased osmotic fragility, and shortened erythrocyte survival. The spleen was not palpable. He had 55 per cent S and 45 per cent C hemoglobin and was considered to represent the sickle-cell-hemoglobin C syndrome. The other two siblings with SC hemoglobin differed in several respects. Neither had ever been seriously ill. The spleen was palpable in both. They were mildly anemic with strikingly microcytic but normochromic red cells. Target cells were numerous. There was marked resistance of the erythrocytes to hypotonic salt solutions. Electrophoresis revealed a greater proportion of C than S hemoglobin. There was no increase in fetal hemoglobin. Red cell survival studies indicated mild hemolysis.

The striking difference between these two parents and their brother with SC hemoglobin, plus findings indicating thalassemia trait in other members of the family, led us to believe they represented the first examples of the anticipated SC thalassemia syndrome.

19. LONG-TERM STUDIES OF IRON OVERLOAD IN DOGS

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Large amounts of iron as saccharated iron oxide or as hemoglobin from transfused red blood cells were given to six dogs to determine the effects of prolonged iron-overload and in an attempt to product hemochromatosis. Intravenously administered saccharated iron oxide was given daily in 100 to 200 mg. doses to a total iron concentration of 0.5 Gm./Kg. in two dogs and 1.0 Gm./Kg. in two other animals. Transfusions of whole blood totaling more than twelve liters were given to each of two dogs to yield concentrations of 0.5 Gm./Kg. hemoglobin iron. At intervals during a seven-year period of observation, blood counts, serum iron, fasting blood sugar, serum bilirubin, bromsulfalein retention, and serum protein determinations were done. Tissue samples for iron analysis and histologic study were obtained by biopsy initially, at six months and three years after beginning iron administration, and at autopsy. Despite concentrations of iron chosen to be comparable to those found in human hemochromatosis, there were no findings in the dogs suggestive of hemochromatosis. Acute toxicity was minimal in spite of greatly elevated serum iron levels, and no laboratory evidence of cirrhosis or diabetes was obtained. Blindness due to retinal degeneration of unknown pathogenesis was the only long-term abnormality noted. Major tissue iron deposits were confined to the liver, spleen, mesenteric lymph nodes, and bone marrow with only a slight sprinkling of hemosiderin in the pancreas and other organs. Although parenchymal iron deposition was seen, the predominant distribution was in the reticuloendothelial cells whether the iron came from hemoglobin catabolism or from saccharated iron oxide. No fibrosis or cirrhosis was produced by the iron deposits in the liver or elsewhere. One of the principal problems in the pathogenesis of hemochromatosis is whether increased tissue iron deposits by themselves eventually produce cirrhosis and pancreatic fibrosis or whether additional factors must be present. The results of these studies suggest that prolonged tissue iron-overload in concentrations comparable to those of human hemochromatosis is well tolerated and in a seven-year period of observation does not of itself produce hemochromatosis in the dog.

20. EFFECT OF MILK AND VITAMIN D PREPARATIONS UPON GROWTH DURING THE PRESCHOOL YEARS

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It is important that growth studies be conducted during the preschool years since socioeconomic differences not apparent at birth are readily demonstrated in children 7 years old. A sample of 63 boys and girls was drawn from the total population of white first graders in a rural county by a method

which insured proportionate representation of all segments of the height continuum. The mother of each child was interviewed in her home to learn the child's intake of milk and vitamin D from birth until he was measured in the first grade. All 63 mothers co-operated willingly, and the interview technique used had been carefully formulated over a period of 19 months and pretested with use of more than 200 informants. The dietary histories served to divide the children into 3 subgroups: (a) 16 children averaging more than 12 oz. of milk daily from ages 2 to 7 and more than 650 I.U. of vitamin D from ages 0 to 7; (b) 20 children with milk intakes of more than 12 oz. and vitamin D intakes of less than 650 I.U. over the same years; and (c) 27 whose milk amounted to less than 12 oz. and vitamin D to less than 650 I.U. on the average. Analysis of covariance was used and the subgroup mean heights and weights adjusted to age 7.16 years were, respectively: 120.9, 118.1, and 116.8 cm.; and 52.6, 48.2, and 47.7 pounds. Differences between subgroup means were significant at the 5 per cent level and combining sexes within subgroups does not appear to have adversely affected the results.

This preliminary study indicates that best growth is produced by daily intakes during the preschool years both of milk in excess of 12 oz. and vitamin D in excess of 650 I.U. Milk intakes of this level are not so successful if unaccompanied by a vitamin D consumption of better than 650 I.U. The children received vitamin D both in fortified evaporated milk and in concentrates. The latter, regularly used by the best growing group, were of such nature that they provided additional vitamin A as well as vitamin D.

21. "INCOMPLETE LEFT BUNDLE BRANCH BLOCK": A FREQUENT NORMAL ELECTROCARDIOGRAPHIC VARIANT

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One hundred healthy young subjects have been studied using an electrocardiographic technique of increased sensitivity (2 cm./mv.), rapid paper speed (75 mm./sec.), with limb leads and leads from sites around the chest at conventional and higher levels, monitored by simultaneous control tracings. The electrocardiograms of 11 subjects demonstrated findings compatible with "incomplete left bundle branch block," that is, absent Q-waves in V_5 and V_6 (Wilson), or slurring of the upstroke of the R-waves in these leads, with or without Q-waves (Sodi-Pallares), and small Q-waves in high precordial right ventricular-type leads.

It is believed that these observations strongly suggest that the interventricular septum of many healthy subjects is initially activated from right to left, indicating the presence of "incomplete left bundle branch block" of such a degree that it can be recognized with available clinical electrocardiographic equipment.

The Q-waves in high right chest leads due to this entity may raise the question of old anterior myocardial infarction. They also may be seen in chest leads from conventional sites (V_1 and V_2) of older subjects with low diaphragms secondary to pulmonary emphysema. Some investigators have interpreted the latter as associated with right ventricular hypertrophy.

22. RECOVERY OF BACILLUS ANTHRACIS FROM THE NOSE AND THROAT OF APPARENTLY HEALTHY WORKERS

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(INTRODUCED BY WILLIAM H. BEIERWALTES, M.D.)

Anthrax is still a hazard to those working with animal hair and hides. A study was undertaken to determine if *Bacillus anthracis* could be recovered from healthy workers in 2 mills using goat hair. Anthrax has occasionally occurred there and *B. anthracis* has been recovered from workroom surfaces. For sampling, workers came directly from workroom to clean room away from contaminated areas. Government employees working elsewhere were controls. After nose and throat swabs had been taken, pharyngeal washings were obtained. Samples were uncovered only the minimum time necessary. Swabs were cultured on blood and brain-heart-infusion agar. Washings were subjected to heat shock, digestion with mycozyme and amylase, and filtration through thin cellulose ester disks, which were placed directly on media. Organisms fulfilling the usual criteria for *B. anthracis* were subcultured and identification was made by virulence tests in mice, with bacteriologic and pathologic confirmation. Identification was further confirmed by lysis with omega anthrax phage. With one exception, the results of virulence and phage tests agreed.

One hundred and one mill workers were sampled. *B. anthracis* was isolated from nasal swabs of 7 and pharyngeal washings of 7. No individual yielded *B. anthracis* from both. All throat swabs were negative. Of 39 subjects working in early, dusty stages of processing hair (pickers, carders, combers), 11 yielded positive cultures. Of 62 working in later stages (drawers, spinners), 3 yielded positive cultures. This difference appears significant ($P < 0.01$). All samples from 43 controls were negative.

It is concluded that *B. anthracis* is present in the nose or throat of some workers using goat hair, at least temporarily after exposure. The hazard appears related to the specific type of work within the mill. Lack of correlation between positive nose and throat cultures may be related to breathing habits. Carriers of *B. anthracis* could represent a serious danger. These findings may explain cases reported in the literature in which family contacts of exposed workers developed anthrax, but the possibility of dissemination of spores via clothing or skin surfaces is not excluded.

23. INACTIVATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN HUMAN ERYTHROCYTES

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An abnormality of glucose-6-phosphate (G-6-P) dehydrogenase activity in erythrocytes of individuals who undergo an acute hemolytic crisis when given primaquine has recently been described (*Science:* in press). The abnormality was demonstrated by coupling G-6-P dehydrogenase with glutathione reductase using dialyzed hemolysate as source of enzymatic activity. Some of the conditions necessary to demonstrate this abnormality have now been elucidated.

Preparation and treatment of hemolysate were done at 4 to 10° C. with exceptions described below. Incubation of whole hemolysate from either normal or primaquine-sensitive erythrocytes for one hour at 10° C. prior to dialysis preserved G-6-P dehydrogenase activity in both hemolysates. Incubation at 37° C. inactivated G-6-P dehydrogenase activity of hemolysate from sensitive erythrocytes. Incubation at 45° C. inactivated G-6-P dehydrogenase activity of hemolysate from both sensitive and normal erythrocytes, the former more than the latter. Glutathione reductase activity was unaffected at these temperatures.

However, when supernatant from either kind of hemolysate, centrifuged at 25,000 × g, was incubated at 37° C. no loss of G-6-P dehydrogenase activity occurred. Sediment from either kind of hemolysate recombined with supernatant from hemolysate of sensitive erythrocytes again caused loss of G-6-P dehydrogenase activity. No inactivation occurred in supernatant from hemolysate of normal erythrocytes when incubated with sediment from hemolysate of sensitive erythrocytes. When sediment and supernatant were dialyzed separately in 0.067 M phosphate buffer prior to incubation, recombined, and then incubated, entirely similar results occurred. When incubated at 45° C., supernatant from centrifuged hemolysate of normal erythrocytes showed no inactivation.

We conclude that inactivation by heat of G-6-P dehydrogenase activity in hemolysates of human erythrocytes requires a stromal factor. This factor is nondialyzable in 0.067 M phosphate buffer. G-6-P dehydrogenase activity from primaquine-sensitive erythrocytes is much more heat labile than that from normal erythrocytes.

24. HOMOZYGOUS HEMOGLOBIN D DISEASE

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The prevalence of the heterozygous form of Hgb. D (Hgb. D trait) in the Negro has been established to be 0.4 per cent. It may be calculated that the homozygous state for Hgb. D should occur in one out of 250,000 Negroes. One such individual has been observed and the syndrome of homozygous Hgb. D disease has been characterized.

The patient, a 40-year-old Negro woman, had always been in good health except for some easy fatigability for ten or more years. Physical examination was within normal limits. She has a light complexion and her features are not typically Negroid. Hematologic studies were as follows: red cells 5,500,000 to 6,500,000 per cu. mm.; hemoglobin 12.5 to 13.5 Gm. per cent; hematocrit 38 per cent; MCV 67 cu. microns; MCH 23 $\gamma\gamma$; MCHC 35 per cent. Leukocyte and platelet counts were normal. Reticulocytes were 1.8 per cent. The erythrocytes appeared somewhat hypochromic and microcytic with slight anisocytosis and poikilocytosis. Target cells comprised 60 to 70 per cent of the red cells. The bone marrow revealed a moderate erythrocytic hyperplasia. The osmotic fragility curve was shifted symmetrically to the right with 10 per cent hemolysis occurring at 0.34 per cent saline and 90 per cent hemolysis at 0.20 per cent saline. Coombs test was negative. Van den Bergh and liver function tests were normal. Serum iron and iron-binding capacity were normal. The hemolytic index was 12. Cr^{51}-tagged red cells disappeared somewhat more rapidly than normal, t/2 being 21 days. Paper electrophoresis studies of the patient's hemoglobin indicated a single component moving in the position of Hgb. S. Sickling of the red cells was absent and geling could

not be induced in a concentrated reduced solution of her hemoglobin. Hemoglobin solubility in 2.58 M phosphate buffer at 25° C. was 1.3 Gm./L. The alkali denaturation test for Hgb. F was normal.

Family studies revealed that the patient's three full sisters have Hgb. D trait while no one of three half-brothers has Hgb. D. Neither parent is living but several of their relatives also lack Hgb. D. In addition to Negro blood, white English and American Indian ancestry are present in the kinship. Although the genetic criteria for homozygosity for Hgb. D cannot be proved, it seems likely that this patient represents an instance of homozygous Hgb. D disease.

26. THE EFFECT OF 5-HYDROXYTRYPTAMINE UPON SMALL INTESTINAL MOTILITY IN MAN

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(INTRODUCED BY LEWIS E. JANUARY, M.D.)

Serotonin or 5-hydroxytryptamine (5HT) has been shown to produce contraction of isolated small intestinal strips of experimental animals; however, little information is available regarding the pharmacologic effects of this substance on the gastrointestinal tract of man. Since patients with the malignant carcinoid syndrome often have diarrhea, it has been suggested that 5HT exerts a stimulating effect upon intestinal musculature.

We determined small intestinal motility in 33 normal volunteers by the balloon-kymograph method. After a suitable control period, 5HT was given intravenously in a single injection. Usually 1 to 2 mg. elicited an intestinal response; however, in six subjects doses up to 3.5 mg. were ineffective. Approximately 30 seconds after administration of 5HT a sharp rise in intestinal tone occurred and mixing waves disappeared. After an average time of 3.7 minutes a gradual return to the baseline had occurred and a period of hypomotility followed. Four subjects had decreased intestinal tone following the administration of serotonin instead of the usual period of increased tone. This resembled the response seen after intravenous adrenalin.

The increase in intestinal tone was accentuated by the prior administration of certain antihistaminic agents which are known inhibitors of monoamine oxidase. Banthine diminished or abolished the rise in tone but hexamethonium was without effect. It appears, therefore, that the intestinal response to 5HT is mediated through the postganglionic parasympathetic fibers. Five-hydroxytryptamine in doses up to 30 mg. failed to produce an intestinal response when given intrajejunally to normal volunteers.

Twelve patients with cirrhosis were studied in a similar manner. The minimal dose of 5HT necessary for an intestinal response was determined in each patient and found to vary from 0.125 mg. to 1.5 mg. Seven of 11 cirrhotics had a typical response when 20 mg. of 5HT was instilled into the jejunum. Two patients with nontropical sprue responded to intraintestinal administration of the drug though the minimal effective intravenous dose was 1 mg. The possible relationship of this hypersensitive reaction to monoamine oxidase deficit will be discussed.

27. INTERMITTENT ALDOSTERONISM IN THE PATHOGENESIS OF FAMILIAL PERIODIC PARALYSIS

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ANN ARBOR, MICH.

A 19-year-old male athlete, who has suffered for years from typical familial periodic paralysis, has been studied extensively from the metabolic and endocrine points of view. Metabolic balances extend over a period of 141 days, in the course of which eleven typical attacks have occurred (spontaneous or induced). Attacks have lasted for as long as 48 hours and have included hourly determinations of the various indices which have been followed.

In addition to the balance data, a large number of bioassays for aldosterone have been carried out in strategic relation to the onset and conclusion of attacks. Other direct indices of adrenal cortical activity have consisted of daily determinations of 17-hydroxycorticoids and 17-ketosteroids. Indirect indices of aldosterone activity included frequent determinations of sodium and potassium in both sweat and saliva, as well as many determinations of urinary pepsinogen. Electrolyte concentrations in muscle and red blood cells were observed during two of the attacks.

RESULTS

1. Familial periodic paralysis is associated with frequent periods of mild elevation of aldosterone secretion during the intervals *between* clinically recognizable attacks.

2. The attack itself is ushered in by a tremendous increase in the secretion of aldosterone, other indices of adrenocortical activity remaining normal.

3. Immediately preceding the clinical attack, retention of sodium occurs. This is followed quickly by retention of potassium, the latter being associated with a sharply falling level of serum potassium. Thus potassium is abruptly sequestered within the body. During most attacks serum sodium rises as serum potassium falls. It returns to baseline values as serum potassium rises and the attack subsides.

4. For several days immediately following an attack, a large diuresis of sodium occurs, followed by a relatively small diuresis of potassium. By this time urinary aldosterone has returned to the preattack level.

The data indicate that familial periodic paralysis is associated with intermittent aldosteronism. It seems likely that an important abnormality in this disease consists of a defect in the extra-adrenal mechanism which regulates adrenal secretion of aldosterone.

28. THE RATIO $\frac{\text{Tm}}{\text{RENAL WEIGHT}}$: AN INDEX OF RENAL SCARRING

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The ratio of the functional tubular mass of the kidney (Tm_{PAH}) to in vivo determined renal weight has been conceived in this laboratory as an index of renal scarring.

$$\text{Renal weight} = \frac{\text{Fick PAH (RBF)}}{\text{N}_2\text{O (RBF)}} \times 100 = \frac{\text{ml. per min.}}{\text{ml. per 100 Gm. per min.}} = \text{Grams}$$

The following evidence, based on studies of this function in 5 normal subjects and 12 patients with renal disease, is presented in support of this thesis. The normal range for the ratio was 0.16 to 0.26. Patients with chronic glomerulonephritis, where Tm_{PAH} is reduced in proportion to weight as nephrons are destroyed by glomerular damage, had normal values. In contrast, individuals with chronic pyelonephritis, where damage is primarily parenchymal and followed by scar formation, showed a reduction of the ratio in all cases. Diabetics, with nephropathy, where damage may be primarily glomerular and/or parenchymal, had values which were either within normal limits or reduced. Patients with benign nephrosclerosis showed an elevation of this relationship.

These findings, with the exception of benign nephrosclerosis, are therefore in accord with the changes which might be expected from the pathologic findings in these entities. In all types of kidney disease these observations are in agreement with previous results relating renal oxygen consumption to Tm_{PAH}.

29. FUNCTIONAL RESIDUAL LUNG VOLUMES AT REST AND DURING EXERCISE IN PULMONARY AND CARDIAC DISEASE

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(Introduced by David P. Earle, M.D.)

Methods currently in use for determining the functional residual volume (FRV) of the lung are applicable only to resting patients. Changes in FRV due to exercise have been inferred from spirograms that show an alteration of the pulmonary midposition but have not been actually measured. The closed circuit helium rebreathing method of Meneely was adapted for both resting and exercise determinations. The spirometer-blower system was modified to reduce the internal dead space and to provide flow rates adequate for exercise determinations, negligible resistance to breathing, and efficient carbon dioxide absorption.

Duplicate determinations were performed on all patients at rest in the upright position and during a 1.4 to 2.0 mile per hour level walk on a treadmill. The largest difference between the 32 pairs of duplicate determinations was 220 ml. or 7 per cent of the average FRV for that pair. FRV decreased on exercise in 4 normal subjects (range 260 to 430 ml. or 7 to 15 per cent). FRV increased during exercise (430 to 730 ml. or 8 to 16 per cent) in four of five patients with severe pulmonary emphysema. The exception had an ab-

normally large tidal volume both at rest (1,255 ml.) and during exercise (1,420 ml.). In one asymptomatic patient with bullous emphysema of the right upper lobe, FRV increased 235 ml. with exercise, and one patient with minimal emphysema showed no change. In five mitral stenosis patients FRV decreased during exercise in two (410 and 420 ml.), increased in one (120 ml.), and did not change in two.

If Stewart's formula is applied to the data of Chapman and Fraser, an increase in pulmonary blood volume of 300 to 600 ml. between rest and exercise can be expected in normals. This approximates the decrease in FRV during exercise observed in our normal subjects. The variation in response to exercise in mitral stenosis patients may represent differences in cardiac function resulting from other valvular lesions or myocardial involvement and their effect on pulmonary blood volume. The diminished pulmonary compliance of mitral stenosis may also influence the change in FRV during exercise. In emphysema, improved intrapulmonary air distribution and an elevation of the pulmonary midposition have been demonstrated during exercise. These factors may partly explain the increased FRV observed in all but one of our patients. Further studies are in progress to separate the relative contribution of the various factors affecting the change in FRV during exercise.

30. EVIDENCE FOR TWO CORTICOSTEROID BINDING SYSTEMS IN HUMAN PLASMA

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17-OH corticosteroids and to a lesser extent 17-OH corticosteroid glucuronides circulate in the blood loosely bound to plasma proteins (J. LAB. & CLIN. MED. **46**: 807, 1955). However, the degree of binding of 17-OH corticosteroids in plasma of cortisone-treated subjects greatly exceeded that found when hydrocortisone was added to plasma in vitro. Investigation of this anomaly has yielded evidence of two independent systems binding corticosteroids.

Dialysis equilibrium experiments with hydrocortisone-4-C^{14} and plasma from hydrocortisone-treated subjects have shown that hydrocortisone binding is a physical process not requiring cellular activity. The remarkable influence of hydrocortisone concentration on the degree of protein binding has been demonstrated in a dialysis equilibrium system consisting of 10 ml. of plasma, 40 ml. of phosphosaline buffer, and varying quantities of hydrocortisone labeled with C^{14}. The ratio of bound steroid to free steroid fell from about 100 to 1 with additions of from 0.5 mcg. to 10,000 mcg. of hydrocortisone. The affinity of plasma proteins for hydrocortisone has been compared using the sample expression $C = \frac{\text{bound hydrocortisone}}{\text{free hydrocortisone x protein (mg./ml.)}}$. With additions of more than 50 mcg. of hydrocortisone to the dialysis system the value of C remained essentially constant. With smaller amounts of hydrocortisone a progressive increase in the value of C occurred.

The ability of structurally related steroids to inhibit the binding of hydrocortisone-4-C^{14} has been examined in a system containing 0.5 mcg. of hydrocortisone-4-C^{14}, 10 mcg. of competing steroid, and 10 ml. of plasma with 40 ml. of buffer. The order of affinity for the binding sites thus demonstrated was hydrocortisone > corticosterone > desoxycorticosterone > 11 β, 17 α 21-Trihydroxypregnane—3, 20 dione > 3 α 11 β, 17 α, 21-Tetrahydroxypregnane—20—one.

CONCLUSION

Human plasma contains two hydrocortisone binding systems. One, probably associated with albumin, has a low affinity for hydrocortisone but is not readily saturated. The other system, predominant under physiologic conditions, has high affinity but is saturated at low hydrocortisone concentrations. The high affinity of this system for hydrocortisone and the decreased affinity of less polar related compounds differentiates this system from that described by Eik-Nes for bovine albumin.

31. PREDETERMINATION OF COMPOSITE ANTIBODY RESPONSE TO INFLUENZA VIRUSES BY VACCINATION WITH APPROPRIATELY SELECTED STRAINS

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The scope of antibody response to monovalent aqueous influenza virus vaccines in three cohorts of the population is determined by the diverse antigenic experiences that each has acquired previously through infection. For example, children whose exposures have been limited to influenza A-prime produce heterologous antibodies after vaccination only to A-prime strains. In contrast, adults successively infected with swinelike, A, and A-prime viruses respond to vaccination with a composite of heterologous antibodies which reacts with many strains of each of the aforementioned three antigenic families of group A influenza virus. A current objective in vaccination against influenza is to produce at all ages high levels of a composite of antibodies which imitates that found in older persons whose resistance to influenza is the greatest. Three approaches to this objective have been investigated. The results of studies on hemagglutinin inhibiting antibody response in children, recruits, and persons over 30 to multistrain aqueous or oily adjuvant vaccines show that high levels of composite antibody can be achieved in each age group after a single vaccination. The superior immunizing potency of emulsified mineral oil adjuvant vaccines was put to test in each of the same age groups and it was shown that with monovalent adjuvant influenza virus vaccines, antibody response did not broaden beyond the confines determined by the previous age specific antigenic experiences of each group. Of greater interest was the finding that children vaccinated one year before with swine or PR8 virus responded now with a composite type antibody when revaccinated with a monovalent adjuvant vaccine containing an A-prime isolate. Thus it has been shown that vaccination, like infection, can condition antibody response to influenza viruses and that by appropriate selection of strains used successively, composite antibody response to a single strain may be predetermined.

32. EFFECT OF HYPOTHERMIA AND THE LYTIC COCKTAIL ON THE SURVIVAL OF CLOSED-CHEST DOGS SUBJECTED TO LEFT ANTERIOR DESCENDING CORONARY ARTERY LIGATION

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Acute coronary occlusion was produced in 41 closed-chest dogs using a two-stage procedure. In the first stage a loose tie was placed around the left anterior descending coronary artery at its origin and the ends of the ligation

brought through the chest wall and buried under the skin. In the second stage 14 days later the ends were recovered, and gentle steady traction applied. The study was controlled by serial ECG and SGO-T determination on all dogs, and by post-mortem examination and injection studies of autopsy material. Surviving animals were sacrificed and studied 21 days after ligation.

In the control group of 21 dogs ligated under light Demerol-Nembutal anesthesia there were 6 survivors for a mortality rate of 71.5 per cent.

Ten dogs were cooled by immersion hypothermia and ligated at body temperature of 30° C. All of these animals died within minutes.

Ten dogs were ligated after infusion with a lytic cocktail consisting of phenegan, thorazine and Demerol. All dogs died; the longest survival was 48 minutes.

33. THE EFFECT OF ETHER ANESTHESIA ON PYRUVATE AND LACTATE METABOLISM

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Inhibition of carbohydrate metabolism in the cell may be responsible for ether narcosis. This possibility has been extensively investigated in tissue slices but relatively few studies have been conducted in man. Our previous studies in man revealed that ether anesthesia caused a marked decrease in glucose tolerance but relatively little change in fructose tolerance. This was interpreted as indicating that ether inhibits specific enzymatic processes.

The present study was designed to determine the effect of ether on pyruvate and lactate metabolism since pyruvate is the final step in the anaerobic scheme of glycolysis and it supplies via acetyl CoA a main source of fuel for the citric acid cycle.

Ten volunteers were given two pyruvate or lactate tolerance tests, one as a control and one during ether anesthesia. The sodium pyruvate (0.12 Gm. per Kg.) or sodium lactate (0.11 Gm. per Kg.) was given intravenously for one hour by a constant infusion pump. Arterial blood oxygen content was followed by an oximeter and serial measurements of blood gases. The results revealed a marked disturbance in tolerance for both pyruvate and lactate during ether anesthesia despite adequate arterial oxygenation. The average rise during the initial thirty minutes of infusion was 1.33 mg. per cent (control), 3.13 mg. per cent (anesthesia) for pyruvate and 8.8 mg. per cent (control), and 21.4 mg. per cent (anesthesia) for lactate. The glucose level fell during the infusion of pyruvate or lactate. The average fall was 8 mg. per cent (control), 16 mg. per cent (anesthesia) for pyruvate and 7 mg. per cent (control), and 26 mg. per cent (anesthesia) for lactate. During thirty minutes of anesthesia without pyruvate or lactate infusion there was a mean rise of 32 mg. per cent for glucose, 6 mg. per cent for lactate, and 0.7 mg. per cent for serum inorganic phosphorus. There was no significant rise (mean 0.06 mg. per cent) in pyruvate. The results will be considered with regard to the possibility that decreased energy production due to impaired pyruvate and lactate metabolism may result in narcosis.

34. MUSCLE ELECTROLYTES AND AMINO ACIDS IN CUSHING'S SYNDROME

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(INTRODUCED BY WALTER H. PRITCHARD, M.D.)

The concentrations of Na, K, Cl, amino acids as measured by the colorimetric ninhydrin method (NRM), and basic amino acids (BAA) have been measured in the plasma and in muscle biopsies from 4 control patients and from 2 untreated Cushing's syndrome patients. The mean values for the normal muscle are 18.5, 39.0, 12.4, 14.4, and 0.83 mM per 100 Gm. of fat-free dry tissue, respectively. In the first patient, the values are 30.4, 37.0, 19.0, 12.1, and 0.37. In the second, they are 20.1, 43.0, 12.4, 16.8, and 0.57. Hypokalemia and alkalosis was present in both. In the first patient, the "(Cl − 1)" space of Darrow is doubled. It is so large as to make it improbable that it measures the extracellular phase of muscle in this patient. Calculated cell Na is not increased in either patient. The observations on muscle Na and Cl confirm those of Eliel and associates. Besides the two reported here, 3 biopsies by Loeb, 3 by Kepler and associates, and 2 by Eliel and associates have been reported in untreated Cushing's syndrome. Only 2, by the last authors, have had significant K depletion when compared to control human muscle. This indicates that alkalosis and depletion of muscle K are not inseparable in the human. The increase in muscle BAA observed in K-depleted rats by Eckel and associates is not observed in these patients.

35. SODIUM DEFICIENCY AND ADRENOCORTICAL HORMONE SECRETION

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Previous investigation has demonstrated that sodium restriction results in hypertrophy of the zona glomerulosa and increased secretion of aldosterone. Although there is evidence which indicates that production of other adrenocortical hormones is unaltered by sodium deficiency, the problem has not been thoroughly studied. For this reason, an investigation of the effects of severe, chronic sodium deficiency on adrenal steroid secretion was carried out.

Young, male Wistar rats were placed on a synthetic diet deficient in sodium. Pair-fed controls received the same diet to which 0.6 per cent sodium chloride was added. Animals from both groups were sacrificed 2, 4, 7, 9, 14, 30, and 60 days after beginning the diets, and serum sodium, potassium, blood hematocrit, and adrenal weights were determined. One adrenal from each rat was incubated aerobically at 37° C. in a buffered medium containing ACTH. After two hours' incubation, steroids secreted were recovered and quantitatively determined by a spectrophotometric technique. This procedure measures all biologically active steroids secreted by the rat adrenal and, thus, represents total hormone production of this gland. In addition, the secretion of individual adrenocortical hormones was determined by paper chromatographic analysis. The remaining adrenal from each rat was used for histologic study.

The results demonstrate that total steroid secretion by the adrenals of control rats increased progressively as the animals grew. Total adrenal steroid production by sodium-deficient animals remained constant throughout the experiment and was significantly ($P = < 0.01$) lower than in controls.

Chromatograms revealed increased secretion of aldosterone by the adrenals of sodium-depleted rats but synthesis of corticosterone and other hormones was

reduced. These changes were correlated with diminished serum sodium concentration and increased size of the zona glomerulosa.

It is concluded from these observations that prolonged sodium depletion results in increased synthesis of aldosterone but the secretion of other adrenocortical hormones is greatly diminished. It is suggested that these changes may interfere with the ability of the animals to withstand stress of other types.

36. NOCARDIOSIS-COMPLICATING DISEASES OF THE BLOOD-FORMING ORGANS WITH BENEFICIAL EFFECT OF STILBESTROL

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Nocardiosis, a relatively rare type of fungus infection, is being reported with increasing frequency. We have seen nocardiosis develop in three patients with diseases of the blood-forming organs. All had received prolonged therapy with antibiotics and steroids, and two had received antimetabolites.

The first two patients, one in an aplastic phase of polycythemia vera, and the second with acute monocytic leukemia in partial remission, failed to respond to 2-hydroxystilbamidine. Curtis and co-workers, in 1952, reported striking effects of another aromatic diamidine, stilbestrol, in two cases of blastomycosis being treated for concomitant prostatic carcinoma. On the basis of this study the second patient was treated with massive doses of stilbestrol (9.2 Gm. over a period of thirty days). The fever, which had been spiking irregularly to 40° C., started falling on the second day of treatment and returned to normal in 2½ weeks. The chest x-rays showed increasing bilateral upper lobe infiltrates with central excavation on the left and bilateral effusion at the onset of therapy. During therapy the parenchymal changes resolved completely, followed by gradual clearing of pleural effusion. The third patient died in the terminal phase of chronic granulocytic leukemia before any effects of stilbestrol therapy could be detected.

Eight strains of nocardia, including the three from these cases, were studied in vitro and all showed a high sensitivity to stilbestrol. Among representative agents previously advocated for the treatment of nocardiosis, sulfadiazine, 2-hydroxystilbamidine, stilbamidine, aminostilbamidine, and propamidine were all studied and showed no inhibitory effect at concentrations of 100 μg/ml., while Aureomycin alone showed an inhibitory effect at concentrations ranging from 25 to 500 μg/ml. Consistent inhibition of all strains was shown with stilbestrol at concentrations of 5 to 10 μg/ml.

Stilbestrol, administered in large doses, deserves further trial in the treatment of nocardiosis.

37. BLOOD PRESSURE STUDIES IN INFANTS WITH AN ELECTRIC MICROPHONE

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(INTRODUCED BY EDWARD MASSIE, M.D.)

It is quite difficult to study the blood pressure variations in infants with use of the conventional auscultatory and palpatory techniques. It was found that the use of an electric microphone proved to be an easy and accurate means of determining the systolic pressure in infants.

In order to determine the accuracy of the electric microphone, the following studies were carried out.

1. Direct intra-arterial pressure determinations in three monkeys were compared with pressures obtained with the electric microphone.
2. Direct intra-arterial pressures of the umbilical arteries of two newborn infants were measured and compared with pressures subsequently obtained by the electric microphone.
3. Blood pressure measurements were made in twenty adults and ten children with use of auscultatory and palpatory techniques and were compared with pressure obtained by the electric microphone.

Excellent correlation was found in the above studies.

The following examples will be cited to demonstrate the practical application of this technique.

1. Two infants, four weeks and six weeks of age, with coarctation of the aorta and patent ductus arteriosus were studied pre- and postoperatively and followed during surgical correction.
2. An infant, sixteen months of age, with an adrenal tumor was followed during surgery and the monitoring of the blood pressure proved to be lifesaving.

38. CARDIAC COMPLICATIONS IN PATIENTS WITH NONTOXIC AND TOXIC NODULAR GOITER AND WITH GRAVES'S DISEASE

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In past studies on cardiac disorders associated with thyrotoxicosis no distinctions have been made usually between the toxic nodular goiter and the diffuse goiter of Graves's disease. Consequently, data on expected frequencies of cardiac complications, expected responses to treatment, etc., are difficult to evaluate. The present study was aimed to appraise the thyroid and cardiac status of 182 patients with nodular goiters (70 toxic and 112 nontoxic) and 51 patients with Graves's disease. An effort was made to determine the following: (a) the incidence of the more serious cardiac complications (other than mere tachycardia and cardiac consciousness), (b) the odds in favor of a nodular goiter being toxic if auricular fibrillation (AF) is present, (c) whether the presence of AF is correlated with the level of the protein-bound iodine (PBI) and with heart disease, and (d) what results might be expected with treatment.

The 70 patients with toxic nodular goiters differed from those with Graves's disease in the following respects: (a) advanced age (average, 65 years), compared with 38 years, (b) much higher incidence of associated organic heart disease (82 per cent), heart failure (58 per cent), and auricular fibrillation (53 per cent) as compared with 21 per cent, 8 per cent, and 12 per cent, respectively, for Graves's disease. Nevertheless, the mean PBI was somewhat higher (13.2 mcg. per cent) in Graves's disease than in toxic nodular goiter (10.0 mcg. per cent). Further analysis indicated that if AF is present with a nodular goiter, the odds are 2:1 that the goiter is toxic and the chances are almost 100 per cent that independent organic heart disease is present. In toxic nodular goiters and in Graves's disease there was no relationship between the level of the PBI and the presence of AF. Also, with adequate treatment of the hyperthyroidism in no more than 25 per cent of the cases will chronic auricular fibrillation spontaneously revert to sinus rhythm, although

improvement in heart failure will occur more frequently. The presence of auricular fibrillation, therefore, is more a direct function of age and accompanying heart disease than a function of hyperthyroidism, although the latter appears to be a precipitating factor.

39. ETIOLOGIC AND CLINICAL STUDIES OF INFECTIOUS MONONUCLEOSIS

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Preliminary results of an intensive investigation of the etiology of infectious mononucleosis will be presented. Throat washings from over 50 patients with clinical infectious mononucleosis have been studied in HeLa cell cultures and over 20 have been studied in a variety of tissue cell types including human kidney, tonsil, conjunctiva, amnion, liver, and lung cells and monkey kidney cells. Several agents have been isolated, 2 of which cause cytopathogenic effects and others which produce no apparent cell destruction but are associated with the appearance on subsequent histologic examination of giant cells, acidophilic intranuclear inclusion bodies, and a characteristic change in the pattern of the cell sheet. The possible relationship of these agents to the etiology of infectious mononucleosis will be discussed as well as the possibility of latent infection of the tissue cells themselves.

A brief clinical analysis of 140 students clinically diagnosed as having infectious mononucleosis will be made. Comparison of 3 groups presented: Group 1 with clinical, hematological, and serologic change; Group 2 with clinical and hematological only; and Group 3 with clinical evidence alone. The need for recognition of serologically negative cases will be made known.

40. METABOLIC EFFECTS OF ARYL SULFONYLUREA COMPOUNDS IN NORMAL MEN AND IN VARIOUS TYPES OF DIABETIC PATIENTS

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ANN ARBOR, MICH.

To elucidate the mechanism of action of sulfonylurea compounds extensive metabolic balance studies and numerous individual testing procedures have been performed before, during, and following administration of BZ-55 and/or Orinase to the following subjects: (a) normal men, (b) middle-aged, obese diabetics, (c) a severe, unstable diabetic, (d) a patient with lipoatrophic diabetes, (e) a totally depancreatized woman, (f) a patient with coexisting diabetes mellitus and Addison's disease, (g) a patient with Cushing's syndrome and coexisting diabetes mellitus, (h) an acromegalic with mild diabetes, and (i) a patient with panhypopituitarism and coexisting diabetes mellitus.

Administration of the sulfonylurea compounds to normal men produces severe hypoglycemia. In normal men and in those diabetic patients who exhibit a blood sugar-lowering response to these compounds we have demonstrated that under their influence there occurs: (1) no significant change in glucose tolerance tests, (2) persistence of both adrenalin- and glucagon-induced hyperglycemia, (3) no consistent change in sensitivity to exogenous

insulin, (4) no significant change in urinary excretion of 17-hydroxycorticoids or 17-ketosteroids, (5) no direct or consistent effect on balances of nitrogen, sodium, potassium, and chloride, (6) no effect on Prednisolone-induced loss of carbohydrate tolerance, (7) no alteration in the metabolic effects produced by Prednisolone, and (8) no evidence of impairment of liver function.

In diabetic subjects who show no appreciable blood sugar-lowering effect by the sulfonylurea compounds there is (1) no effect on insulin sensitivity as measured by insulin tolerance tests and (2) no difference in insulin requirement whether the diabetes is controlled with insulin plus sulfonylureas or with insulin alone. BZ-55 cannot be substituted for insulin in the pancreatectomized human.

When Orinase is administered, a carboxylic acid derivative of this compound is excreted in urine as a major excretory product. This compound exerts no hypoglycemic effect.

BZ-55 caused a great depression of thyroidal uptake of I^{131} in one patient.

The data indicate that the sulfonylurea compounds (1) do not suppress the pituitary-adrenal system, (2) do not antagonize the peripheral effects of adrenal corticoids, and (3) do not block the hyperglycemic effects of glucagon and adrenalin. No evidence of potentiation of insulin activity could be demonstrated. The blood sugar-lowering property of these compounds is via another mechanism.

41. PROTEIN METABOLISM IN EXPERIMENTAL NEPHROSIS

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This study was designed to answer the following questions regarding protein metabolism in the nephrotic syndrome: (1) Is there an increased rate of synthesis of serum albumin? (2) Is this rate maintained constant during the progression of the syndrome? (3) What effect has cortisone therapy on this rate? (4) Is this a maximal rate of protein synthesis?

Nephrosis was produced in rats by the administration of 6-dimethylaminopurine 3-amino d-ribose, an aminonucleoside related to the experimental antibiotic puromycin. The disease was characterized by the development of ascites, proteinuria, hypoalbuminemia, and hypercholesterolemia within a two-week period. The in vitro incorporation of C^{14} glycine into the soluble protein of liver slices was considered an index of serum albumin "synthesis" since this soluble protein was previously shown to be immunologically identical with serum albumin.

In nine control animals, the specific activity (counts/min./100 μg of protein) of the soluble liver protein was found to be 231.8. In nine nephrotic animals the specific activity (S.A.) was 410.3 indicating a significantly increased rate of glycine incorporation. There was no increase in S.A. of protein in nephrotic animals until the level of serum albumin was significantly depressed, following which there was no striking further change in the S.A. Cortisone treatment depressed the rate of protein synthesis in these nephrotic animals and was not associated with any amelioration of the syndrome. In an effort to answer the question regarding maximal rates of synthesis, control rats were subjected to acute plasmaphoresis experiments. Twenty-four hours later, the S.A. of their liver protein was found to be 612.5. This markedly enhanced incorporation of glycine into protein indicated that the accelerated rate of protein synthesis observed in nephrotic animals was not the maximal rate of which the organism is capable. The factors possibly involved in preventing the nephrotic animal from reaching maximal rates of protein synthesis will be discussed.

42. TREATMENT OF HYPERTENSIVE EMERGENCIES: A STUDY OF THE COMPARATIVE EFFECTS OF PARENTERAL RAUWOLFIA PREPARATIONS

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(INTRODUCED BY ELLARD M. YOW, M.D.)

The usefulness of parenteral Reserpine in the treatment of hypertensive crises has established a need for further evaluation of new forms of Rauwolfia which are available for parenteral administration. Ten patients have been studied under controlled conditions for the hypotensive and other pharmacologic effects following the administration of four parenteral Rauwolfia compounds: Reserpine, Raunormine, Recanescine, and Serpentine.

After the administration of Reserpine at an average dose of 6 mg. intramuscularly, there was decline in the upright mean blood pressure from 147 mm. of mercury to 113 mm. of mercury ($p < .001$) with an average onset of effect of 120 minutes and a duration of 15 hours. There were no side effects other than mild somnolence.

After the administration of Raunormine, there was decline from 150 mm. of mercury to 115 mm. of mercury within 79 minutes with a duration of 15 hours. Four of the patients complained of significant dizziness and nausea. Somnolence was less marked. The average dose was 16 mg.

After the administration of Recanescine at an average dose of 17 mg., there was no significant change in blood pressure, but there were marked adverse pharmacologic effects consisting of flushing, angina, weakness, headache, and nervousness.

After the administration of Serpentine at an average dose of 18 mg., there was a slight reduction in blood pressure from 146 to 130 ($p > .01$) within 114 minutes with a duration of 12 hours. There were no significant side effects.

The results indicate that Reserpine is still the drug of choice with the least number of side reactions in the treatment of hypertensive patients in crisis. The techniques as described may be applied to the evaluation of other parenterally usable drugs in the treatment of hypertensive disease.

43. EFFECT OF EPINEPHRINE AND L-NOR-EPINEPHRINE UPON THE ISOLATED BEATING RAT HEART

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The isolated rat heart, beating in saline, is capable of filling and emptying itself repeatedly. During systole, pressures in the left ventricle of 11 to 84 mm. water were recorded; during diastolic filling by suction, negative pressures of 2 to 24 mm. water were recorded. The purpose of this study was twofold: (1) to determine the effect of l-nor-epinephrine upon the isolated heart, (2) to determine whether a drug-induced change in the force of systole produces a corresponding change in the force of diastole.

Twenty-seven rat hearts were studied. The beating heart was quickly excised and placed in saline or lactate-Ringer's solution. Simultaneous left ventricular pressures and electrocardiograms were recorded by means of a cathode ray oscillograph. In 19 hearts, the effect of adding 5 gamma l-nor-epinephrine to the bath was observed; in 8 hearts, the effect of addition of 5 gamma epinephrine to the bath was observed.

RESULTS

Mean heart rate before l-nor-epinephrine was 70.6/minute and was 97.5/minute after l-nor-epinephrine. The mean increase of 27 ± 6.4 was significant, $p < 0.001$. The product of heart rate times systolic pressure was 1,727 before and 3,679 after l-nor-epinephrine. The increase in cardiac work by this calculation of 1,952 ± 657 was significant, $p < 0.02$. There was also a significant increase in diastolic cardiac work after nor-epinephrine, $p < 0.05$.

After epinephrine, the mean heart rate increased from 83 to 140/minute. The increase of 57 ± 11.4 was significant, $p < 0.001$. The increase in work after epinephrine was not significant, $p < 0.1$.

A regression equation indicated significant relationship between change in systolic and in diastolic peak pressures in the entire group of hearts, $p < 0.01$.

It is concluded that l-nor-epinephrine is capable of exerting a direct effect upon the isolated beating rat heart, increasing its rate and force of contraction in both systole and diastole. It is also concluded that there was a significant relationship between the force of systole and the force of diastole in this experiment.

44. COMPARISON OF THE EFFECTS OF ORAL METHYLTESTOSTERONE, 19-NORTESTOSTERONE, AND 17-METHYL-19-NORTESTOSTERONE ON SERUM LIPIDS AND LIPOPROTEINS

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Recent interest in nortestosterone and C-17-substituted derivatives is derived from the suggestion that they possess protein anabolic properties, but are largely devoid of androgenic effects. The ability of a steroid to reduce the serum concentration of high density $-S_{1.21}$ 0-12 ("alpha") lipoproteins has proved a sensitive and reliable index of androgenicity.

Eleven subjects received a total of 13 courses of methyltestosterone. Dosage ranged from 25 to 200 mg./day. Significant ($p < 0.01$) reduction in $-S_{1.21}$ 0-12 lipoproteins always followed methyltestosterone administration, while $-S_{1.21}$ 25-40 ("beta") or $-S_{1.21}$ 25-70 lipoproteins increased significantly in most instances. The reverse was the case when methyltestosterone was discontinued. The values of the ratios $-S_{1.21} \frac{0\text{-}12}{25\text{-}40}$ or $\frac{0\text{-}12}{25\text{-}70}$ always varied significantly with the $-S_{1.21}$ 0-12 changes. The cholesterol and lipid phosphorus concentrations varied together with the lipoprotein fraction showing the change of greater magnitude.

Seven subjects received 20 to 200 mg./day of nortestosterone. Significant reduction in $-S_{1.21}$ 0-12 lipoproteins and in the values of the ratios $-S_{1.21} \frac{0\text{-}12}{25\text{-}40}$ and $\frac{0\text{-}12}{25\text{-}70}$ resulted. The lower density $-S_{1.21}$ 25-40 and 25-70 lipoproteins showed no significant changes as a result of nortestosterone administration in this dosage. Serum cholesterol levels failed to change significantly, although lipid phosphorus values fell significantly in 5 of 8 courses.

Four subjects were treated with 20-200 mg./day of methylnortestosterone, and significant reduction in $-S_{1.21}$ 0-12 lipoproteins and reduced values for the ratios $-S_{1.21}\frac{0\text{-}12}{25\text{-}40}$ and $\frac{0\text{-}12}{25\text{-}70}$ resulted in each instance. Reciprocal changes in $-S_{1.21}$ 25-40 and 25-70 lipoproteins were significant in each subject except one who received only 20 mg./day for 10 days. Serum cholesterol values varied in the direction of the lipoprotein class manifesting the greater change, but usually were not significant.

Metabolic balance studies utilizing these steroids indicated all possessed unequivocal protein anabolic properties.

It is concluded that these three agents differ only quantitatively with respect to their effects on lipoproteins. The order of decreasing androgenicity would appear to be: methyltestosterone, 17-methyl-19-nortestosterone, and 19-nortestosterone. Methylation enhances the androgenicity of nortestosterone.

45. SOME LABORATORY AND CLINICAL EXPERIENCES WITH A NEW ANTIBIOTIC, VANCOMYCIN

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WITH THE TECHNICAL ASSISTANCE OF EDWARD WARREN (BY INVITATION), AND DOROTHY ROSE (BY INVITATION)

Vancomycin, a predominantly bactericidal antibiotic, is relatively stable, soluble in water, active against gram-positive bacteria, especially micrococci, shows no cross resistance with other antibiotics, is relatively low in toxicity, and induces resistance in micrococci slowly and in slight degree. Its activity is not significantly altered by pH changes or by the presence of serum.

Studies with 112 strains show that micrococci are uniformly sensitive to the action of vancomycin; 110 were inhibited by 2.5 μg or less per milliliter. The same or only slightly greater amounts were required for total killing effect. Its bactericidal effect on nonhemolytic streptococci and clostridia varied more.

Animal-protection tests with an experimental micrococcal infection in mice indicated that 25 mg. per day per kilogram of body weight was sufficient to protect against death; 100 mg. was required to protect against gross lesions. Vancomycin had no effect on an experimental infection in mice with *Toxoplasma gondii*.

With oral and intravenous administration of the drug the feces became odorless or nearly so, clostridia disappeared, and enterococci were reduced in number or disappeared. The gram-negative flora persisted.

An agar-diffusion method was used for vancomycin assays. Following single intravenous injections of 500 mg., average serum values of 33, 4.3, 2.8, 1.5, and 0.7 μg per milliliter were obtained 1 minute and 3, 6, 12, and 24 hours after the injection. After multiple intravenous doses every 6 hours, average serum values of 8.5 μg were found (range: 6.4 to 13.8 μg). Large amounts of vancomycin are excreted in the urine; at 24 hours average values

of approximately 100 μg per milliliter remained. Therapeutic amounts appear in the bile. With multiple intravenous doses, 10 to 110 μg per gram appear in the stool; with multiple oral doses of 500 mg., usually several thousand micrograms per gram appear in the stools (range: 80 to 24,000). No vancomycin was detected in the serum and little in the urine following oral administration.

Vancomycin diffuses through the pleural, pericardial, peritoneal, and synovial membranes; it does not cross uninflamed meningeal membranes.

Preliminary clinical experiences with micrococcal infections have been good.

46. EFFECTS OF PYRAZINAMIDE ON SERUM AND URINE URIC ACID

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(INTRODUCED BY EDMUND B. FLINK, M.D.)

Patients being treated with pyrazinamide (3 Gm./day) for tuberculosis were found to exhibit hyperuricemia. Serial 24-hour urine and serum urates were measured in patients and controls, using a uricase ultraviolet spectrophotometric method. Pyrazinamide itself did not interfere with this method.

In six male patients, serum urate ranged from 9.4 to 13.6 mg. per cent (ten controls—3.6 to 6.1 mg. per cent) and urine excretion ranged from 140 to 395 mg./day (ten controls—400 to 740 mg./day) on ordinary hospital diet. Serum urate was 8.2 mg. per cent and excretion 220 mg./day in one female patient.

Pyrazinamide was administered to three males after control periods. Urinary excretion decreased precipitously to extremely low levels and then rose slowly into the above subnormal ranges. Serum urate rose rapidly and remained elevated. Simultaneous creatinine determinations and other clinical renal function tests revealed no abnormality, except that PSP excretion ability was depressed in some patients. On discontinuing pyrazinamide, the urate abnormalities reversed promptly, but an excess of urates was excreted for about one week.

One patient with no previous gout developed acute gouty arthritis shortly after pyrazinamide was discontinued. However, his serum urate remained elevated. Pretreatment levels were not available. It appears probable that he had asymptomatic gout and the induced abnormalities simply precipitated his first attack.

Increased tubular reabsorption of urates appears to be involved, but alternatives, such as altered ultrafiltrability of urates are not excluded. Decreased urate excretion persisted after many months of pyrazinamide therapy, apparently reflecting decreased production. This may be a physiologic response to elevated serum levels, but may represent more fundamental disturbance in urate metabolism. The observed phenomena present potential tools for further study of uric acid metabolism and gout.

47. SURGICAL HYPOPHYSECTOMY IN THE TREATMENT OF DIABETIC NEPHROPATHY

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Eleven patients with advanced vascular complications of diabetes mellitus of the Kimmelstiel-Wilson type have been subjected to surgical hypophysectomy in an attempt to alter the otherwise progressive course of their vascular

disease. Prior attempts to produce complete destruction of pituitary function by means of heavy x-ray therapy had met with uniform failure. This experimental approach was originally considered on the basis of present knowledge of the Houssay phenomenon in the hope that amelioration of the diabetic state might be accompanied by a parallel improvement in the vascular complications.

With one exception, all patients tolerated the surgical procedure without difficulty. One patient, a dwarfed 21-year-old boy, died within 48 hours postoperatively. Two other patients died 6 and 14 weeks, respectively, after returning home, as the result of severe, prolonged hypoglycemic reactions. The remaining 8 patients are ambulatory, feeling well with normal or near-normal vigor, and two have returned to work. Five are blind, as a result of their original disease, to the extent that they are unable to work.

Insulin requirements have decreased strikingly in all cases with reductions in dosage varying from 50 to 80 per cent, although in no instance has it been possible to eliminate the use of insulin completely. All patients have been maintained on replacement therapy consisting of cortisone from 25 to 50 mg. daily in divided doses, and l-thyroxine 200 μg daily. Sex steroids have not been administered regularly, but testosterone has been used in several of the male patients. Striking improvement in the retinopathy has occurred uniformly, and measurable return of vision has been noted in several individuals. Hypertensive blood pressure levels have returned to normal and renal function as evaluated by routine clinical methods has improved.

Histological study of the biopsy material and metabolic studies performed both pre- and postoperatively will be discussed.

48. ELECTROCARDIOGRAPHIC OBSERVATIONS DURING CLOSURE OF INTERVENTRICULAR SEPTAL DEFECTS WITH THE ADJUNCT OF ACETYLCHOLINE-INDUCED CARDIAC UNREST

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A series of 8 children with interventricular septal defects, all with severe pulmonary hypertension, have been operated on by the open-heart technique, while extracardiac circulation was maintained by the De Wall-Lillehei pump-oxygenator apparatus. During the cardiotomies, the hearts were stopped by the intracoronary injection of acetylcholine. This maneuver permitted the surgical repair to be done in a perfectly dry and quiet field. Continuous electrocardiographic monitoring during the procedures provided the records herein reported.

Mechanical systole was promptly abolished in all cases following the intracoronary injection of 10 mg./k of acetylcholine. Complete electrical standstill was observed during the arrest in some cases although occasional wide QRS complexes with preservation of slow, regular P waves were seen in others in spite of the absence of visible contraction activity. Manipulation of the heart occasionally caused a weak idioventricular contraction. As soon as the interventricular defect was closed, the heart was resuscitated by remov-

ing the clamp across the ascending aorta which permitted perfusion of the coronaries with oxygenated blood. Wide QRS complexes began to appear regularly within 5 to 10 seconds after the clamp was removed. These complexes became more rapid and less bizarre, but with A-V dissociation remaining. Strong cardiac contractions were usually present at this time. Sudden reversion to sinus rhythm occurred in from one-half to three minutes after sinus rhythm was established and, very rapidly, the electrocardiogram returned to the original pattern.

In 6 cases, an effective normal sinus rhythm was established, but 2 of these patients subsequently expired because of complications apparently unrelated to the induced cardiac arrest. Ventricular fibrillation was intractable in one patient both before and after the induced arrest, and in another patient the recovery phase was complicated by recurring fibrillation.

49. STUDIES ON THE HEPARIN-LIKE EFFECT OF DEXTRAN SULFATE

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We have had occasion to investigate the anticoagulant effect of dextran sulfate (Dexulate-Glaxo, London, England), a synthetic sulfated polysaccharide. The material (supplied in 5 c.c. ampules, 1,000 units/c.c.) was administered to a total of 12 patients. Eight had normal clotting mechanisms and received a single intravenous dose of 5,000 units. Four patients who had thromboembolic disease (3 deep femoral thrombophlebitis and 1 probably pulmonary infarct) were given the drug in a constant intravenous infusion. Subsequent determinations were made of the Lee-White clotting time in all cases, the Quick one-stage prothrombin time in 9 cases, the two-stage prothrombin time (Ware-Seegers Method) in 1 case, the modified Owren (Ware) prothrombin time in 2 cases, Factor V (proaccelerin) in 3 cases, Factor VII (proconvertin) in 1 case, and fibrinogen in 1 case.

The clotting time was uniformly prolonged. This effect was marked (from one and a half to ten times the control value) during the first hour after the single dose and persisted into the second hour on only two occasions. When the drug was administered by continuous infusion it was possible to control the clotting time by regulating the speed of infusion. The Quick one-stage prothrombin time was promptly and significantly prolonged. This effect would last from 6 to 12 hours following the single dose. Factor V activity was markedly reduced in all three cases; this paralleled the effect on the Quick one-stage prothrombin time. The modified Owren (Ware) prothrombin time, two-stage prothrombin time, Factor VII activity, and fibrinogen levels were unaffected by the drug. No hemorrhagic or other toxic effects were noted in these short-term studies.

50. A CLINICAL BIO-ASSAY OF ANALGESIC ACTIVITY

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D-Propoxyphene Hydrochloride (alpha d-2-propionoxy-4-dimethylamino-1,2, diphenyl-3-methyl-butane hydrochloride) has been given orally for the

relief of chronic pain to patients with any illness. Five medications in identically appearing capsules were used: (1) a blank; (2) codeine phosphate, 32.5 mg.; (3) d-Propoxyphene, 32.5 mg.; (4) codeine phosphate, 65 mg.; and (5) d-Propoxyphene, 65 mg. Three samples of each were prepared with different labels and supplied to the investigators on a blind basis. The investigators received fifteen unknowns to be tested and had been told they would receive a blank, d-Propoxyphene, and codeine phosphate.

Each of the samples was given for twenty-four hours. At the end of each twenty-four hours, the patients were interrogated as to the number of hours of slight, moderate, and severe pain endured. Each hour of slight pain was scored 1, each of moderate pain 2, and each of severe pain 3. Thus, fifteen pain scores were obtained on each of 101 patients.

The patients were unable to distinguish equal doses by weight of d-Propoxyphene and codeine phosphate. A measurable increase in the discomfort was reported by these chronically ill patients when 32.5 mg. was substituted for 65 mg. or when the blank was substituted for either analgesic. Codeine phosphate in doses of 65 mg. produced a significant number of undesirable gastrointestinal side effects not seen with the other medications.

Nine of these 101 patients were also seen hourly by nurses and time-effect curves determined. For the first two hours after administration the patients indicated pain relief with the blank equal to that with the analgesics. A measurable increase in discomfort was reported on the third, fourth, fifth, and sixth hours when the blank was substituted for the analgesic. The two analgesics were undistinguishable in intensity or duration of pain relief.

Clinicians in almost every field of medicine prescribe codeine. The development of a drug which is equally analgesic and produces fewer undesirable side-effects is, therefore, of general interest.

51. THE APPLICATION OF SEQUENTIAL ANALYSIS TO THERAPEUTIC STUDIES WITH PHARMACODYNAMIC AGENTS

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The relatively new statistical technique of sequential analysis, as developed by Wald and associates for use in industry, has been applied to clinical data to evaluate the effects of numerous pharmacodynamic agents on several widely different body processes. The use of sequential analysis requires primarily that there be a firm body of base-line (control) data and suitable randomization of the therapeutic trials. When these requirements were met it was possible to make valid appraisals of the action of new agents in human subjects in a short time and after relatively few observations.

Observations were controlled by the administration of placebos by the double blind technique and according to standard randomizing procedures. Five body processes susceptible to alteration by pharmacodynamic agents were selected for study: (1) ipecac induced nausea and vomiting which yielded data containing a binomial distribution (yes or no answer), (2) urine output in response to the administration of several diuretic agents which

yielded numerical data with a one-sided alternative, (3) gastric acid secretion in response to stimulating and inhibiting agents, (4) blood glucose levels following the administration of hyper- and hypoglycemic agents, (5) pupillary size following exhibition of miotic and mydriatic agents. The latter three series of experiments yielded numerical data with a two-sided alternative. In all, 19 pharmacodynamic agents were tested in 635 observations on 420 human subjects.

In the case of each agent a decision was possible regarding its effectiveness in less than 25 observations, usually less than 15 observations and often less than 10. The validity of the inferences arrived at by sequential analysis was in each instance confirmed by continuing the experiment and then applying standard statistical analysis (usually the t-test).

The findings from tests of pharmacodynamic agents on each of the diverse areas of body function identify sequential analysis as an important tool in therapeutic research.

52. RED CELL LIFESPAN AND IRON TURNOVER IN PATIENTS WITH HYPOXIA DUE TO PULMONARY EMPHYSEMA

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Wilson and co-workers (Arch. Int. Med. **88**: 581, 1951) have demonstrated that patients with pulmonary emphysema do not show the hematological adaptation to hypoxia characteristic of residents at high altitude. The reason for this failure has not been established, however. Wilson and associates showed that it was not related to carbon dioxide retention and suggested that it might be secondary to chronic infection.

Sixteen patients with severe pulmonary emphysema and arterial oxygen saturation below 92.0 per cent were studied. Pulmonary function studies were characteristic of pulmonary emphysema; vital capacity 2.17 ± 0.36 L.; one second vital capacity 28.0 per cent ± 15 per cent; maximum breathing capacity 31.2 ± 18.1 L.; residual volume 3.41 ± 1.13 L.; total lung capacity 5.73 ± 1.30 L.; nitrogen content of alveolar air at end of 7 minutes of breathing oxygen 3.8 ± 0.70 per cent; arterial oxygen saturation 84.7 ± 7.9 per cent; arterial carbon dioxide content 55.9 ± 7.20 vol. per cent; and pH 7.39.

The hematological data showed hematocrit 54.1 ± 5.6 per cent; hemoglobin 16.8 ± 1.1 Gm. per cent; red blood cell count 5.27 ± 0.53 × 10^6 per cubic millimeter; M.C.V. 103 ± 5.7 cubic microns; M.C.H. 32 ± 2.1 $\mu\mu$g; M.C.H.C. 31 ± 2.1 per cent; reticulocyte count 0.95 ± 0.37 per cent; serum iron 129 ± 47 μg per cent. The Cr^{51} red blood cell half-life was 30.0 ± 7.6 days; red cell mass 48.0 ml. per Kg. of body weight; Fe^{59} disappearance 78 ± 46 minutes; and the Fe reappearance 5.4 days. None of the group reached the predicted hemoglobin level for their degree of hypoxia and the mean hemoglobin for the group was 88 per cent of the predicted. The normal red cell lifespan and normal iron turnover indicate that the failure to adapt fully to the low oxygen saturation is not related to iron deficiency or hemolysis. Moreover, the finding of normal red cell lifespan and normal rate of Fe^{59} disappearance contrasts with some reports of the hematological findings in the anemia of chronic infection. The explanation of the phenomena described must therefore lie in the responsiveness of the bone marrow.

53. THE ANTAGONISTIC ACTION OF MAGNESIUM AGAINST TETRACYCLINE IN VITRO AND IN VIVO

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During the past 3 years American and English investigators have discovered that certain bivalent cations antagonize various biologic effects of chlortetracycline and oxytetracycline. Thus magnesium reverses the inhibiting action of chlortetracycline upon oxidation in rat liver mitochondria; manganese reverses inhibition of nitro-reductase of *Endamoeba coli* by chlortetracycline; magnesium, iron, aluminum, and others antagonize, in varying degrees, the antibacterial action of these tetracyclines against certain strains of microorganisms.

We have investigated 2 separate manifestations of this phenomenon. The first is the antagonism by magnesium of that tetracycline carried over in subcultures in sufficient quantities to inhibit growth of sensitive staphylococci. Addition of $MgSO_4 \cdot 7\ H_2O$ to cultures containing 1 to 5 μg per ml. of tetracycline abolishes its antibacterial action in a ratio of approximately 3,000 μg $MgSO_4 \cdot 7\ H_2O$ to 1 μg tetracycline.

It is well known that aluminum salts given along with chlortetracycline lower the antibiotic blood level. The mechanism of this action has never been elucidated. It seemed possible that the effect might be part of the more general property of antagonism of bivalent cations against tetracyclines, and that magnesium might act similarly in the gastrointestinal tract. Five-tenths or 1 Gm. tetracycline was given orally to 10 patients. A serum sample was obtained just prior to the dose and 1 and 4 hours after. Two days later the patient was given the same dose along with 15 Gm. $MgSO_4$. In every instance there was a marked diminution in blood level of tetracycline after its administration in conjunction with $MgSO_4$. The average levels showed a fourfold decrease. The administration of castor oil to 5 of the patients resulted in no diminution of blood level, demonstrating that the effect was not due to the cathartic action of $MgSO_4$.

Similar experiments with orally administered tetracycline, performed in 10 rhesus monkeys, confirmed the findings in man. When tetracycline was given to monkeys intramuscularly and $MgSO_4$ by mouth, there was no diminution in tetracycline blood levels.

These studies demonstrate that magnesium combines with tetracycline in 2 different biologic situations. Whether the combination is a simple chemical one or perhaps one of chelation has not been demonstrated.

54. STUDIES ON THE GROSS METABOLISM OF STRONTIUM^{85} IN MAN

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Strontium^{90}, a beta particle-emitting radioisotope (T ½ 27-30 years) produced during fission reactions has been identified as one of the "fallout" products from the detonation of nuclear weapons. Strontium^{90}, being a long-lived, beta particle-emitting, bone-seeking radioelement, will induce bone tumors in the experimental animal in a manner similar to that observed in human radium poisoning. The need for data concerning the metabolism of

strontium in the human is patent. Strontium^{85}, a cyclotron-produced, non-injurious, gamma-ray emitter (T ½ 65 days) has been used by this group to study the metabolism of strontium in man.

Twelve patients were studied under controlled metabolic conditions, each receiving a total calcium intake from all sources of 1 Gm. per day. Strontium^{85} chloride was administered orally as a single dose that varied between 10 and 50 μc. Urine and fecal samples were collected and aliquots counted in a well-type scintillation counter. Estimations of total body retention were done daily by suitably counting the entire patient in a shielded room employing a large scintillation crystal placed 1 meter from the patient.

Seven of twelve patients received an oral dose of 10 μc and none retained more than 8 per cent of the original dose nine days after ingestion. The maximal retention was shown by one patient who received 30 μc and was estimated to have a body burden of 14 per cent on the forty-eighth day. The other three patients, who had doses ranging from 20 to 50 μc, all fell within the previously mentioned 8 per cent body retention range. Not more than 20 per cent of the orally administered dose was excreted in the urine and one nephrotic patient excreted only 1 per cent via this route. Fecal excretion was high for the first several days, and then fell precipitously, suggesting that the early high level of fecal excretion represents Sr^{85} unabsorbed from the gastrointestinal tract. Urinary excretion and body retention assume a linear relationship to time on a log-log plot. Continuing studies will make possible the derivation of a power function for the excretion of strontium, thus making more accurate the assessments of possible fission product hazard.

55. THE EFFECT OF ORAL ETHANOL ON THE HEPATIC GLUTAMIC PYRUVIC AND GLUTAMIC OXALACETIC TRANSAMINASE IN THE RAT

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The effect of alcohol was tested on four groups of rats: (1) those receiving 20 per cent alcohol as the sole source of fluid, and on an ad libitum diet (10 rats); (2) those receiving water and pair-fed with those from Group 1 (10 rats); (3) those receiving water, pair-fed with Group 1, and isocaloric by means of fat supplements (10 rats); (4) those receiving water and fed ad libitum (12 rats).

The diet was adequate with respect to lipotropic factors and vitamins, which also were present in the drinking fluid. One-half the animals were sacrificed after 8 weeks, and the remainder after a further 17 days of halved food intake and consequent weight loss. Glutamic pyruvic (GPT) and glutamic oxalacetic (GOT) transaminase determinations were carried out in duplicate on the livers and brains.

After 8 weeks, the mean hepatic GPT activity was significantly lower in Group 1 than in Group 3, in terms of dry weight, and than Group 4, in terms of dry weight and nitrogens. This difference was enhanced by starvation, becoming significant between Groups 1 and 2 also. It was significant between Group 1 and any of the other three groups in the starved and nonstarved animals taken together, in terms both of dry weights and nitrogens. Upon starvation, there was also a significant increase in hepatic GOT activity in Groups 1 and 2.

These changes were not reflected in the transaminase determinations on the brains. As the nitrogen contents of the livers were very similar in the various groups, the differences were independent of the extent of any fatty changes.

The results show that alcohol causes a reduction in hepatic GPT activity that is enhanced by starvation and is indicative of a direct action of alcohol on the liver. The metabolic significance of these findings will be discussed.

56. RETROLENTAL FIBROPLASIA AND THE CENTRAL NERVOUS SYSTEM

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(INTRODUCED BY ROBERT L. JACKSON, M.D.)

Retrolental fibroplasia is a retinal-vascular disease manifesting itself at a specific stage of retinal-vascular growth and development. Oxygen breathed in high concentration is unquestionably the most potent noxious agent to the susceptible retina both in man and in animals. Since the retina is an anterior outpouching of the brain, any process affecting the retina is likely to affect the brain. This paper reports a study designed to establish the susceptibility of the central nervous system to damage from hyperoxemia.

High concentrations of oxygen given pregnant mice are associated with resorption near term pregnancy, stillbirth just before term, and severe abnormalities in the kits when given at term. The defects are delay in hair appearance, growth failure, and severe abnormalities in motor performance. When high concentrations of oxygen are given only after twenty-four hours after birth, these defects are not observed.

Histologic abnormalities of the brain are found only in litters exposed to high concentrations of oxygen before twenty-four hours after birth. The lesions are perivascular, noninflammatory, and characterized by a central hyalinized blood vessel.

Histologic abnormalities of the retina, as reported by others, are found in mouse kits exposed to high concentrations of oxygen between the second and the seventh days after birth.

These results may be compared with clinical experiences with fetal rubella, and are parallel with the cyanide experiments with molting grasshoppers. Various noxious agents may disturb the organizers of rapidly growing tissues in specific organs at specific stages of development.

In mice, hyperoxemia at one atmosphere in mouse kits is associated with histologic and physiologic abnormalities in the brain if given before twenty-four hours after birth, and in the retina if given from twenty-four hours after birth to the seventh day after birth.

57. SIGNIFICANCE OF INCREASED URINARY PEPSINOGEN (UROPEPSIN) EXCRETION IN DUODENAL ULCER AND DURING ACTH ADMINISTRATION

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(INTRODUCED BY SIBLEY HOOBLER, M.D.)

To determine whether the mechanisms of increased uropepsin excretion due to ACTH were the same as those responsible for the increases in patients with duodenal ulcer, pepsinogen clearances were measured simultaneously with gastric pepsin secretion in 5 normal men before and during 6 days of ACTH-gel administration, as well as in 6 ulcer subjects with high uropepsin excretion.

In the normal controls and in the ulcer subjects good linear correlation ($r = +0.82$) existed between urinary and plasma pepsinogen. The mean pepsinogen clearance in ulcer patients (36 ± 19 L./24 hr.) did not differ significantly from that in normals (44 ± 14 L./24 hr.). ACTH administration, however, invariably produced a steep, sustained rise in urinary pepsinogen excretion without change in plasma pepsinogen, the mean clearance increasing to 65 ± 29 L./24 hr. Thus the increased uropepsin excretion in ulcers resulted from elevated blood levels of pepsinogen with a normal renal clearance, whereas the increased uropepsin excretion during ACTH occurred with a fixed plasma pepsinogen and was due to an increased renal clearance of pepsinogen.

The differences between pepsinogen clearances and GFR probably imply some glomerular impediment to excretion as well as some tubular resorption of pepsinogen (M. Wt. 44,000), as is the case with albumin (M. Wt. 47,000). The positive correlation between endogenous creatinine and pepsinogen clearances in the normals ($r = +0.68$), and increases in both by ACTH ($p < 0.01$), though proportionately different (13 per cent and 48 per cent, respectively), suggested that the increases of uropepsin by ACTH may be due partly to increased glomerular filtration of pepsinogen. The uropepsinuric effect of phloridzin (Peczenik) suggests, however, that tubular resorption plays some part in regulating uropepsin excretion.

The relation between gastric pepsin secretion and uropepsin excretion is erratic—ratios vary from 100:1 to 3,000:1, even in the same subject, and various substances may affect one independently of the other. This is not surprising, since at least 3 factors may be responsible for elevation of uropepsin excretion, namely: (a) increased peptic cell mass (e.g., long-standing duodenal ulcer), (b) reversal of secretion gradient of peptic cells resulting in elevation of plasma pepsinogen, and (c) increased renal clearance of pepsinogen as produced by ACTH.

58. ACUTE ALTERATIONS OF SODIUM SPACE IN MAN

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(Introduced by E. B. Reeve, M.D.)

A continuous infusion technique has been employed to measure the volume of distribution of Na^{22} (sodium "space") in 100 normal male subjects. The method results in a stable measurement for time intervals extending from 30 to 240 minutes. Plasma sodium concentration remained constant and urinary Na^{22} excretion was negligible. Sodium "space" measured in this manner is primarily limited to extracellular distribution and the mean value expressed as percentage of body weight was 22.7 ± 3.4. This method has also been used to measure induced acute changes of sodium "space." After a 30- to 60-minute control period, 26 subjects received injections of 9α-fluorohydrocortisone (1-3 mg.). Of this group 19 subjects exhibited increases in sodium "space" ranging from 4 to 54 per cent. Under similar experimental conditions, 11 of 15 subjects receiving Pitressin (0.01-10 units) showed increases in sodium "space" ranging from 9 to 83 per cent. In both groups no correlation was apparent between the dose of the drug and the magnitude of response. In six subjects hydrocortisone exhibited a similar effect but with less regularity. Sodium "space" remained constant following the injection of insulin (9 subjects), glucagon (9 subjects), 50 per cent glucose (5 subjects), 10 per cent glucose (14 subjects), and normal saline (6 subjects). Alteration of position in 11 subjects resulted in striking and consistent effects

on sodium "space." When position was altered from standing to supine, sodium "space" increased, whereas when position was changed from supine to standing sodium "space" decreased. In 4 of these subjects, sodium "space" was measured three consecutive times, the third measurement being made after the subject had returned to his initial position. In these experiments, the final "space" measurement returned toward the initial value. The average increase in sodium "space" from standing to supine position was 63 per cent, while the average decrease from supine to standing position was 24 per cent. Changes in sodium "space" due to position were unaffected by the application of a tight abdominal binder or elastic stockings.

59. STUDIES ON THE ADMINISTRATION OF ATROPINE IN HUMANS

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As a control study for the use of atropine in organic phosphorus insecticide poisoning, 50 experiments were carried out on a group of human volunteers. One and 2 mg. doses of atropine were administered subcutaneously and the state of hydration was varied to include no fluids, 200 ml. of fluids every hour, and 400 ml. every hour. Posture included supine and sitting positions. A control group received saline subcutaneously. Studies were made of the changes in salivary flow and salivary electrolytes, of the changes in urinary volume and urinary electrolytes, and of changes in the serum electrolytes, proteins, and blood cholinesterase and hematocrit. The pronounced drop in salivary flow following atropine was associated in most instances with a significant rise in salivary electrolyte concentrations. With the forcing of fluids, there was a tendency for the salivary flow to return more rapidly toward normal. Symptoms included headache, unsteadiness, hyperirritability, fatigue, and a feeling of indifference. Serum changes were not present in all individuals. However, when present they appeared to be significant and were most marked in the nonhydrated group. These changes will be discussed in respect to fluid shifts. The red cell cholinesterase was frequently depressed. In the nonhydrated groups there was a decrease in the urinary volume and sodium concentrations, and an increase in urinary potassium and phosphorus concentrations. Expressed as meq./minute there was a decrease in sodium and chloride excretion and increases in potassium and phosphorus excretion. With hydration these changes were less pronounced. These changes will be discussed in relation to fluid exchange, and in relation to atropine effect on patients exposed to anticholinesterase agents.

60. THE RELATIONSHIP OF BLOOD GLUCOSE LEVEL TO THE RENAL CLEARANCE OF ENDOGENOUS AND EXOGENOUS PHOSPHATE

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(INTRODUCED BY GORDON MEIKLEJOHN, M.D.)

The renal clearance of both endogenous (C_{PO_4}) and exogenous labeled phosphate ($C_{P^{32}O_4}$) has been studied in 13 normal men. Utilizing standard renal clearance techniques with creatinine as an indication of glomerular

filtration, a sensitive and consistent parallel relationship between alterations in the serum glucose level and C_{PO_4} and $C_{P^{32}O_4}$ was observed. The $C_{P^{32}O_4}$ exceeded the C_{PO_4} from 30 to 86 per cent in all but 2 subjects.

The rise in the C_{PO_4} occurred early after the infusion of glucose and was noted in 9 of the 13 subjects to occur in the first 10-minute period. By the third 10-minute clearance period, all subjects showed a definite increase. For example, increases of 5, 41, 50, and 64 per cent in the serum glucose caused the C_{PO_4} to increase 39, 100, 157, and 174 per cent, respectively, following ingestion of 100 Gm. of glucose by mouth. Peak C_{PO_4} values seldom exceeded 40 ml./min., and were obtained with serum glucose levels of 122 to 188 mg. per cent. Continued increases in the serum glucose levels resulted in no further rise in C_{PO_4}. Analysis of the data indicates that the increase in the C_{PO_4} and $C_{P^{32}O_4}$ is due to the decreased tubular reabsorption of phosphate brought about by the reabsorption of the increased load of filtered glucose.

Because of the differences in the C_{PO_4} and $C_{P^{32}O_4}$, observations were made after the infusion of a buffered phosphate solution. Data obtained after increasing these clearances by the above method indicated that a portion of the endogenous plasma PO_4 is handled differently by the kidney than exogenous phosphate. Thus the "true" clearance of phosphate is represented by the labeled exogenous PO_4.

61. SERUM ELECTROPHORETIC STUDIES OF PATIENTS WITH A RECESSIVELY INHERITED MUSCULAR DYSTROPHY

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(INTRODUCED BY ARTHUR C. CURTIS, M.D.)

Electrophoretic analysis was carried out on the serum of 13 related patients with recessively inherited muscular dystrophy. The serum of the parents of these patients was also studied in an attempt to find some abnormality by which the carrier state of this disease might be recognized.

Electrophoresis was carried out by the Tiselius technique, using Veronal buffer (pH 8.6, ionic strength 0.1 M). The 13 patients studied showed clinical manifestations of muscular dystrophy. Of these 13 patients, 11 showed an abnormal resolution of the α_2 globulin area. The other electrophoretic areas were found to be normal. The 2 remaining patients showed no abnormalities in the electrophoretic pattern.

No clinical manifestations were observed in the parents of these patients. However, electrophoretic analysis of the serum of all living parents showed the same poorly resolved α_2 globulin area that was noted in the patients.

It seems possible, therefore, that the genetic carrier state of this type of muscular dystrophy may be shown by electrophoretic analysis of the serum proteins.

62. PYELONEPHRITIS AND HYPERTENSION IN UNILATERAL RENAL DISEASE

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The pathologic diagnosis made upon 71 kidneys removed surgically during the decade 1945 to 1954 and the clinical records of these patients form the basis of the present study. The investigation was made to define further the

frequency and significance of pyelonephritis among a group of patients with predominantly unilateral severe renal disease; to observe the relationships between hypertension and pyelonephritis among these patients as compared with other unilateral kidney lesions; and to note the effect of nephrectomy upon the course of hypertension which was present before operation.

Chronic pyelonephritis, exclusive of tuberculous pyelonephritis, was present in 41 (57.7 per cent) of the specimens. It was the sole anatomic diagnosis in 4 cases (5.6 per cent). The over-all incidence of hypertension was 30 per cent. Among the patients with an anatomic diagnosis of chronic pyelonephritis, 14 (35 per cent) had hypertension including 3 of the 4 patients with pyelonephritis alone. Among 30 patients without pyelonephritis, 7 (23 per cent) were hypertensive. The greater incidence of hypertension with pyelonephritis was not statistically significant except for the small group of patients in whom pyelonephritis was the sole anatomic diagnosis. In addition to chronic pyelonephritis, the one group also contained significantly more patients with hydronephrosis or nephrolithiasis, and the group without pyelonephritis was significantly weighted by patients with renal carcinoma or tuberculosis. The frequency of hypertension associated with these lesions was similar regardless of whether or not pyelonephritis was present. Azotemia, indicating bilateral renal insufficiency, was present in only 6 patients, among whom 3 had pyelonephritis and hypertension.

The prognosis for improvement of the hypertension after nephrectomy was significantly better when the pathologic lesion was primary pyelonephritis or hydronephrosis and pyelonephritis, in which case 5 of 9 patients with adequate follow-up were improved and only 2 became worse. The prognosis was significantly poorer when the hypertension was associated with nephrolithiasis and pyelonephritis, in which case none of 4 patients improved and 3 became worse.

63. RESPONSES OF NONTOXIC GOITERS TO THYROTROPIN

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The response of the 3-hour thyroidal uptake of I^{131} and the serum protein-bound iodine (PBI) to a single injection of thyrotropin (TSH) has been studied in 37 patients with nontoxic diffuse goiter and 20 patients with nontoxic nodular goiter.

Of the group with diffuse goiter 20 had responses within the normal range, and 10 of those with nodular goiter had normal responses. The remaining 27 patients showed abnormal responses to thyrotropin with respect to I^{131} uptake or PBI or both. Some showed no rise in I^{131} uptake but a normal rise in PBI; some showed no rise in PBI but a normal rise in I^{131} uptake; and some showed no response in either I^{131} uptake or PBI. In some of these the initial I^{131} uptake was elevated, in others it was normal.

These findings indicate that approximately 50 per cent of patients with nontoxic goiter have abnormalities in thyroid function which can be demonstrated by the TSH test. The abnormalities may be of several types, including disturbances in the ability to store or release thyroid hormone as well as disturbances in its formation.

64. RELATIONSHIP OF SICKLING PHENOMENON TO BLOOD OXYGEN CONTENT AT VARIOUS SITES IN THE BODY (STUDIED BY VENOUS CATHETERIZATION) AS CONTRASTED WITH IN VITRO SICKLING

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(INTRODUCED BY JAMES A. CAMPBELL, M.D.)

A series of 10 venous catheterizations was performed on 6 patients with sickle-cell disease and two patients with sickle cell-hemoglobin C disease. Determinations of the per cent oxygen saturation of hemoglobin, whole blood pH, and the number of sickled cells were made on venous samples of blood obtained from the jugular, hepatic, and renal veins, the coronary sinus, the right atrium and ventricle, and the pulmonary artery as well as from the femoral artery.

In each patient there were fewer sickled cells in the arterial sample than in any venous sample. However, in the different patients there was no correlation between the arterial oxygen saturations (which varied from 68 to 86 per cent) and the per cent of sickled cells. From 2 to 16 per cent sickled cells were found in the arterial blood in the patients with sickle disease and only from 0.6 to 2.0 per cent were present in the arterial blood of the patients with S-C disease.

The number of sickled cells in the venous blood samples from patients with sickle disease ranged from 10 to 32 per cent, whereas the patients with sickle cell-hemoglobin C disease had 1 to 6 per cent sickled cells. There was no correlation between oxygen content and the number of sickled cells in the various venous samples obtained from a single patient, those obtained at repeat catheterization of a given patient, or those from various individuals. Venous blood oxygen saturations varying from 22 to 73 per cent were encountered.

The rapid infusion of 2.5 meq./Kg. of 0.85 M sodium bicarbonate failed to influence the number of intravascular sickled cells.

These findings were remarkably different from the in vitro studies wherein changes in oxygen saturation of blood in the same range as in the in vivo studies were accompanied by large changes in the number of sickled cells. These studies suggest that the in vivo sickle phenomenon is not comparable to in vitro sickling and that factors other than oxygen content have important roles in the sickle process.

65. THE EFFECTS OF AMPHENONE IN TWO PATIENTS WITH HYPERADRENOCORTICAL STATES

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WITH THE TECHNICAL ASSISTANCE OF A. BENNETT, B.A. (BY INVITATION), E. HEIDRICK, B.A. (BY INVITATION), AND L. EASON, B.A. (BY INVITATION)

Amphenone B is a stilbene compound which has progestational activity and alters the function of the adrenal cortex and thyroid in animals and man (Hertz and associates: J.C.E.M. **16**: 705). This report concerns the effects of Amphenone in two patients with adrenal cortical hyperfunction.

The first was a 30-year-old woman with congenital adrenal hyperplasia and typical adrenogenital syndrome. Her urinary 17-ketosteroid excretion was controlled by intramuscular hydrocortisone which was continued throughout the present study. ACTH was given for ten days prior to Amphenone. During this time, 17-ketosteroid and 17-hydroxycorticoid excretion rose to peaks of 69 and 71 mg./24 hours, respectively. ACTH was continued during and for three days following Amphenone administration. Five grams of Amphenone was given intravenously on two successive days. Coincident with Amphenone administration, 17-hydroxycorticoid excretion fell to very low levels and did not rise, despite continued ACTH. 17-ketosteroids were unaffected. Results of pregnanetriol determinations will be discussed.

The second patient had Cushing's syndrome caused by a large malignant adrenal cortical tumor with hepatic metastases. Urinary 17-ketosteroid and 17-hydroxycorticoids were about equally elevated. During x-ray therapy to the adrenal area, steroid excretion was increased and qualitatively changed. At first the 17-KS/17-OH ratio was less than control levels and then became greater. Five grams of Amphenone was given intravenously for two days, followed by oral dosage of 3 to 8 Gm. per day. The 17-hydroxycorticoid excretion was markedly reduced, but 17-ketosteroid readings were increased up to fivefold over control levels. On the last three days of Amphenone therapy, 20 mg. TSH was given daily, but PBI and I^{131} uptake remained very low.

Significance of these results and of complications of Amphenone therapy will be discussed.

66. THE INFLUENCE OF A BRAIN EXTRACT ON CHOLESTEROL METABOLISM

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Oral administration of an extract of mammalian brain to human subjects has previously been shown to have an hypocholesteremic effect which resembles in intensity and breadth the action of beta sitosterol. Unlike sitosterol, this extract does not precipitate with digitonin or give color with the Lieberman-Burchard reagent, so that its effects on cholesterol metabolism are more susceptible of study.

In a normocholesteremic patient on a constant fat diet, 500 μc of C^{14} acetate was administered intravenously and the relative rate of cholesterol synthesis derived from the maximum (4-hour) rise in the specific activity of serum-free cholesterol. Eighteen days later, 30 Gm. per day of brain extract was begun, and the specific activity of the serum cholesterol fell abruptly, demonstrating an increased turnover of serum cholesterol. Before conclusion of the treatment period, a second dose of 300 μc of C^{14} acetate was administered. Allowing for the difference in the level of serum cholesterol, which had fallen from 216 to 174 mg. per cent, and the difference in dose administered, the cholesterol synthesis as indicated by the specific activity of the free serum cholesterol at four hours and 24 hours after acetate administration was unchanged as compared with control levels.

Three-day collections of feces were extracted with alcohol exhaustively, and aliquots analyzed by the techniques of Mosbach and associates for digitonin-precipitable material and bile salts. The digitonide was further analyzed by a technique developed in this laboratory for the proportions of cholesterol and coprosterol present.

The dry weight of feces tended to be increased by the administered brain extract and with this was an increase in excretion of cholesterol, coprosterol, and bile acids. The bile acid/cholesterol ratio tended to decrease with con-

tinuing administration of the brain extract; the percentage of sterol present as coprosterol was the same during control and treatment periods except for a reduction during the first week after starting the extract.

Sterol excretion in the feces is definitely enhanced by this hypocholesteremic agent. The means by which this may be achieved will be discussed.

67. EFFECT OF TOLYL-SULFONYLUREA (ORINASE) ON NET SPLANCHNIC GLUCOSE PRODUCTION (NSGP) IN MAN

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(INTRODUCED BY EDWARD H. REINHARD, M.D.)

Results of previous studies suggest that orinase affects certain enzyme systems of the liver and that part if not all of its hypoglycemic action is a result of a reduction in glucose output by the liver. However, no direct measurements of hepatic glucose production in man or the intact animal have been reported.

The effect of 50 to 75 mg./Kilo. of orinase orally on the arterial blood glucose concentration and NSGP of 4 control and 3 elderly, moderately diabetic subjects was studied. In the control subjects the blood glucose fell 27 ± 4.8 mg. per cent (S.E.) ($p < 0.01$) at forty minutes after orinase from a fasting level of 86 ± 2.2 mg. per cent. This was accompanied by a 50 ± 7 per cent ($p < 0.01$) reduction in the total NSGP during the first 40-minute period. After forty minutes both the arterial glucose levels and NSGP showed a variable response.

In the 3 diabetic subjects the fall in arterial blood glucose concentration was more gradual, in accordance with previous observations. At the end of two hours a fall in the blood glucose of 23 ± 5 mg. per cent was accompanied by a 69 ± 12 per cent reduction in total NSGP during this time. Orinase exerted no effect on hepatic blood flow, BSP clearance, or splanchnic oxygen consumption as measured in 2 of the control and in 2 of the diabetic subjects.

Femoral A-V glucose differences in 2 normal and 1 diabetic subjects did not change from the fasting values during orinase hypoglycemia. Although these observations do not warrant conclusions regarding the effects of orinase on peripheral glucose utilization, they do suggest that no significant part of the fall in NSGP was due to an increased removal of glucose by those "peripheral" tissues drained by the portal vein. The best explanation for our findings is a reduction in true hepatic glucose production.

68. GASTRIC SECRETORY STIMULATION: EFFECTS OF PHENYLBUTAZONE, HISTALOG, ACTH, ADRENAL STEROIDS, AND RESERPINE IN MAN

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The importance of HCl in the pathogenesis of peptic ulcer necessitates identification of gastric secretory stimulants and possible activators of the disease. The present study investigated the effect of several potential "ulcerogenic" agents upon basal secretion in man and the role of vagal and adrenocortical mechanisms.

Phenylbutazone, an antirheumatic compound, increased gastric acidity in 30 of 57 studies with single doses of 200 to 600 mg. orally, and in 10 of 32 tests with 50 to 400 mg. intramuscularly. Similar increases occurred in pa-

tients with vagotomy. The absence of significant eosinopenia and the increased acidity after phenylbutazone orally in patients with bilateral adrenalectomy suggested independence of the effect from adrenocortical stimulation. Control observations with water, saline, sodium salicylate, and colchicine were negative. Acetylsalicylic acid stimulated acidity but not when given at neutral pH. Histamine and histalog evoked tremendous outputs of HCl, without significant eosinopenia, in normal individuals, patients with peptic ulcer, and patients with bilateral adrenalectomy.

Single doses of corticotropin intramuscularly and cortisone orally did not increase gastric secretion. Basal secretion also was unchanged in 9 of 10 patients with ulcerative colitis receiving ACTH and adrenal steroids for long periods. In one case, acid output increased temporarily and then returned to control levels despite continued ACTH therapy.

Reserpine, 1.0 mg. orally daily for 12 weeks, increased basal gastric secretion in one of eight subjects. The volume of secretion and concentration of HCl rose markedly following reserpine intravenously (1.0-2.5 mg.) in normal persons, patients with peptic ulcer, and patients with vagotomy. Eosinophile counts did not diminish significantly. Corticotropin and hydrocortisone intravenously had little or no effect, whereas reserpine intravenously in the same patients stimulated large outputs of HCl. The stimulation of gastric secretion by reserpine may be related to increased liberation of histamine from cellular depots; the possible role of serotonin is yet to be evaluated. These studies indicate that certain newer therapeutic agents may stimulate basal gastric secretion markedly in man in spite of interruption of the vagal mechanism and with no demonstrable adrenocortical stimulation.

69. GASTRIC PEPSIN STUDIES WITH RADIOACTIVE ALBUMIN

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(INTRODUCED BY WALTER L. PALMER, M.D.)

The concentration of pepsin in basal (fasting) and stimulated (Histalog, insulin) gastric juice has been measured by a new method using I^{131}-labeled serum albumin. Gastric juice is incubated with the serum substrate. The undigested protein is precipitated with trichloracetic acid. Enzyme concentration is determined by the radioactivity of the supernate.

The fractional gastric analyses demonstrated the continual presence of pepsin in association with free hydrochloric acid. Not infrequently, however, fairly high concentrations of pepsin were noted despite the prolonged absence of HCl. Histalog apparently stimulated the release of pepsin. In several instances of basal achlorhydria, pepsin appeared before free acid could be detected in the juice.

Various anticholinergic agents were tested for their effect on the volume, free acid, and pepsin concentration of the gastric content. Although the volume and acidity may be suppressed, the concentration of pepsin often was unchanged. In other instances, after the disappearance of free acid, pepsin decreased gradually, suggesting selective innervation of the vagus. Radiation to the gastric fundus for duodenal ulcer decreased or eliminated the free acid; however, the pepsin concentration occasionally was unchanged.

Patients with duodenal ulcer may produce large amounts of pepsin, sometimes as much as 600 mg. of pepsin in the volume secreted over a two-hour period. Insulin stimulated large increases of pepsin. Vagotomized patients

may show a decrease in the output of acid and pepsin, and, by the insulin test following complete vagotomy, there may be prolonged periods of gastric secretion without hydrochloric acid or pepsin.

These observations with radioactive albumin suggest that the secretion of pepsin may be dissociated from free acid; that basal secretion of pepsin may be fairly uniform; that the vagus may consist of different fibers which, by moderate or partial blockade, may decrease the amount of free acid and, by more powerful blockade, also may suppress completely the output of pepsin. Roentgen irradiation to the stomach and anticholinergic drugs do not necessarily decrease the output of pepsin, though gastric acidity may be lowered.